

The best global warming treaty yet

Disappointment with the draft global warming treaty agreed last week in New York should not get out of hand. What the world needs is a comprehensive deal and a lasting one. Last week was a good start.

Is it preferable to win a battle or the war of which it is probably a part? That is what the suddenly dispirited people who have laboured for years, not just weeks, on the design of a draft treaty on global warming should now be asking. The circumstances are easily related: in preparation for next month's conference on environment and development in Rio de Janeiro, negotiators in New York have been trying to hammer out the draft of a treaty to restrain the emission of greenhouse gases. Now, many spirits have been dashed because hopes of winning an agreement spiked with specific emission limits seem to have been torpedoed. Specifically, the goal of returning individual countries' greenhouse gas emissions at the end of this decade to those obtaining in 1990 appears in the draft treaty as an "aim", not a requirement. Epithets such as "sell-out" fill the air.

The despondency is misplaced. Indeed, there is every reason to believe that the draft treaty is not only the most rigorous that could have been won at this stage, but that it is preferable to any other there might have been — at this stage. The explanation of that is simple. First, the United States would not have signed a treaty including numbers for emissions limits, but may well agree to "aim" at the abatement of global warming (next month should tell). Which is preferable, that the world's largest artificial producer of carbon dioxide gas should agree to the principle of abatement, or that the rest of the world should band together to call the United States a black sheep, but stomaching its carbon dioxide production unrestrained? That would have been a pyrrhic victory at best. Now there is at least a chance of making progress.

Agreement

Second, there are several greenhouse gas producers probably ready to sign anything on which others at Rio may agree, but which cannot be relied upon — at this stage — to deliver what they promise. Russia is the most conspicuous, for it has other more urgent obligations (such as survival) on its mind. The other members of the Commonwealth of Independent States are in the same case, as are Yugoslavia and, to a lesser degree, the states of Central Europe, China, India and even Greece (which cannot regulate its cash flow, let alone its greenhouse gases). What purpose would be served by a treaty that, from the outset, was acknowledged by its signatories to be leaking like a sieve? How quickly would it be disregarded even by its well-organized members?

Despondency

Despondency over the seemingly milk-and-water treaty emerging from New York is also rightly evoked by a recognition of the intellectual tasks that lie ahead. Two stand out, of which the more technical is that of deciding how to balance the effects of the competing nuisances of carbon dioxide, methane and chlorofluorocarbons (CFCs), the principal greenhouse gases except for water vapour (which is part of the problem, not the solution). The obvious difficulty is that the economic consequences of getting the balancing equations wrong are potentially so great that misjudgement in the middle of a long recession (see *Nature* 355, 1; 7 May 1992) could itself be catastrophic. Has anybody calculated the likely consequences of the popular carbon tax for the prospects of resumed economic growth?

Still more serious is the problem of equity. Plainly, the fiat of the rich countries cannot deny the poor the right to follow paths of economic development that are similar to their own. That seems to have been accepted in New York by the draft treaty's exemption of poor countries from all obligations except those of measuring greenhouse emissions and brooding about their containment. For the time being, that is fine, and meanwhile there will be the prospect of grants from the World Bank to help with environmental problems. But what will happen further down the road, when the likelihood that the Earth's capacity for tolerating increased emissions is exhausted (as some say it is already)? In a world in which the right to emit greenhouse gases equates directly with economic well-being, will the flow of aid transfers persist? Or population growth be left off the agenda (as, apparently, is the intention for Rio)?

This must not be mistaken for a cynical prediction of an awful future, but is rather a recognition of the unacknowledged courage of those who work for a treaty on global warming. They are working for a world in which nations' social policies and economic aspirations all hang together crucially, and in which all governments are so enlightened that they accept the obligations that follow without cavil — or resignation from the treaty. Utopia? Not necessarily, but people may be forgiven for supposing that it will take some time to come about.

But is not the problem of global warming much more urgent? The global warming lobby says that the longer abatement is delayed, the greater will be its eventual cost. That cannot be denied. Argument legitimately centres only on the timing of significant climatic change and on the costs



of whatever mixture of abatement and adaptation is adopted, by accident or design. The calculations of the timing of change are hugely uncertain, those of cost much more so, but it is not unreasonable to suppose that it will be half a century, give or take a decade or so, before the surface of the Earth goes seriously awry. This journal's consistent opinion over several years is that it will take far longer to build a durable greenhouse regime than to remove the remaining uncertainties in the predictions.

That is why, even at this stage, the fine print of the first stab at a global warming treaty needs close attention, and at two levels. The technical issues are taxing enough. Can a country's records of fossil fuel imports and production be taken as a reliable proxy of carbon dioxide emission, for example? Does a burning oil-well count against a country's target and, if there is a carbon tax, who pays tax to whom? Although wood-burning may be greenhouse-neutral over the lifetime of forest trees, what happens when energy-restricted communities begin burning wood more quickly than they should (which is inevitable)? What is to be said and done about methane, a quickly increasing greenhouse gas whose sources are only poorly understood? If agriculture in the form of animal husbandry and rice-growing proves to be the culprit, will there be a democratic government willing to impose restrictions on its farmers in the interests of a global warming treaty? And what, in any case, are the current predictions of the climatic consequences of current atmospheric concentrations of greenhouse gases?

What the treaty most urgently needs is a way of providing a running commentary on issues such as these. The obvious model is the UN Scientific Committee on the Effects of Atomic Radiation (UNSCEAR), which has over three decades provided periodic authoritative reviews of current understanding of the hazards of peaceful and warlike nuclear power. It has the advantage that its members are appointed by governments, but function independently of them, thus retaining their professional authority. But global warming is a more urgent problem than even UNSCEAR's, whence the need that the new committee (UNSCOG?) should report once a year at least (and be sufficiently equipped for that). Its main task, as the years go by, would be the progressive refinement of the goals at which the signatories of a global warming treaty should aim. One of the few certainties of this field is that the numbers will change continually, as time passes, in one direction or the other.

The second class of fine print is that dealing with the political matters that must inevitably be covered by a global warming treaty. Industrialized countries will say that the less of that there is, the better. But there must be some. The rich countries must at least acknowledge that they must bear the brunt of at least the earlier phases of restraint. So much appears to have been agreed in New York last week. The extent to which, and the price at which, they are obliged to compensate poor countries for forgoing rational but atmospherically damaging developments (such as burning the Amazon Basin) is a matter for bargaining, on which the two sides are still far apart. The really contentious issues —

population restraint, surrender of sovereignty to monitoring organizations and the conversion of aid funds into those administered by the World Bank's Global Environmental Facility — are as yet untackled. Can the International Negotiating Committee make a useful start on them? That is what matters most. Meanwhile, those weeping over a supposed sell-out in New York should put their energy into making sure that the Rio treaty is ratified by the 50 signatories needed to get the show on the road. □

Science on hit-list

The White House and Congress vie with each other in their ignorant attacks on science.

US PRESIDENT George Bush has started an election year budget fight with Congress that has resulted in a contest over which side can make scientific research look silliest (see page 103). In the process, each side is simply showing its ignorance.

It began in March when Bush (the Republican politician) denounced the Democratic Congress for its "perks, privilege, partisanship and paralysis" and declared that he could save the US taxpayers \$4,000 million if Congress would only give him the authority to cut specific items from the budget. The big item in Bush's savings plan, which would take back money that has already been approved for spending in what is charmingly known as a "line-item recision", was the Seawolf submarine. (Cancelling the two submarines on the drawing-board would reduce the budget by \$3,000 million.) The other \$1,000 million would come largely from science projects. The Bush hit-list included research on eastern filbert blight, storage of Vidalia onions, fungus-resistant celery and seedless table grapes.

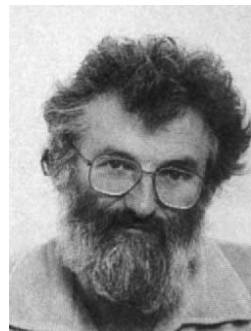
The list provided a field day for Democrats who countered with their own list of expendable silly science, including a study of "holism in psychobiology in the twentieth century" and "affective bases of person perception", each approved by peer review.

This exercise in political absurdity, which is likely to come to naught, reveals how far outside the mainstream science still is. Despite a strong Office of Science and Technology Policy in the White House and a host of science committees in the Congress, nobody seems to have thought twice about perpetuating the myth that scientific research (selected by peer review, as was each of the challenged projects) is at once self-indulgent foolishness on the part of scientists and, in any case, beyond the comprehension of ordinary citizens.

Bush and the Congress should be embarrassed by their narrow-minded approach. If either the President or the Congress wishes to challenge the wisdom of the peer-review system (which they certainly have a right to do), they should ask substantive questions about the validity of the science, not attack it just because it sounds silly — although in the case of holism in twentieth-century psychobiology the Democrats may be on to something. □

Sweeter offer by Wellcome keeps genome scientist in Britain

London & Washington. Possibly the largest genome research centre in the world may be established in Britain as the result of discussions that are taking place between the Medical Research Council, the Wellcome Trust and John Sulston, leader of the project to map and eventually sequence the genome of the nematode worm *Caenorhabditis elegans*. The talks end speculation that Sulston and his collaborators on the *C. elegans* project might go to work for the



John Sulston

private sector, in particular a gene sequencing company being created by US industrialist Frederick Bourke (see *Nature* 355, 483; 1992).

Sulston's group had a three-year grant, for £2 million a year (US\$3.5 million), that ended last month, and the MRC has asked the government to renew its funding of the project. That money, if awarded, would follow the nematode project into the new centre.

The Wellcome Trust, one of the largest

medical research charities in the world, has yet to decide on the size and details of its contribution, but Michael Morgan, the trust's programme director, said that the sum would be comparable to what is currently being spent on the nematode project. The two funding bodies have set up a joint working party to further plans for the centre. It is hoped that, when established, it will also attract money from elsewhere in Europe.

The core of the centre will be a sequencing facility based on techniques developed by Sulston's group at the MRC's Laboratory of Molecular Biology (LMB) at Cambridge. The group now produces around one megabase of sequence a year for *C. elegans*, and the intention is to extend this to five megabases a year at the new centre, an increase Sulston says is achievable with his group's existing techniques. Such an output would make the centre one of the most prolific in the world.

As well as stepping up activity on the nematode sequencing, the core facility will also work on the human genome, which is a particular interest of the Wellcome Trust. The intention is to make the centre more than a sequencing factory, with other senior scientists joining Sulston to pursue their own research involving selected regions of the human and other genomes. Expertise on

informatics will also have a place at the centre, although initial computer support will come from LMB.

It is not known where the centre will be based, although Cambridge is a strong contender. The strong international links that have been a feature of the *C. elegans* project are expected to carry over into the new arrangement. Robert Waterston, a geneticist at Washington University in St Louis, Missouri, is Sulston's principal collaborator. He too is hoping to enlarge his laboratory considerably, and has applied to the US National Institutes of Health for a major increase in funding. Although the two researchers plan to continue a scaled-up *C. elegans* collaboration, Waterston is not planning to expand into human genome research.

Although the formation of a UK centre would rule out a move by Sulston and his co-workers into the private sector, the possibility of commercialization is not excluded from their plans. "There may be spin-off companies as a result of the focused activity at the centre, but there is unlikely to be any direct commercialization," said Michael Morgan. Direct commercialization would breach the Wellcome Trust's status as a charity.

Ian Mundell & Christopher Anderson

Russians reassure India on rocket contract

New Delhi. Russia last week told India that it will fulfil its contract to supply cryogenic rocket engines despite having been told by the United States (see *Nature* 356, 732; 1992) that the contract violates an international agreement to prevent the spread of nuclear technology.

In a visit made to clarify his country's stance on the rocket deal, Gennady Burbuli, Russian secretary of state, said that "neutral international experts" will be examining the contract to establish whether it conforms to the Missile Technology Control Regime (MTCR). Burbuli did not name the experts who will examine the contract or specify when it would be done.

Although it is not a signatory to the regime, Russia has undertaken to abide by its provisions. Burbuli told Indian officials that Russia is also morally bound to fulfil its commitments under the contract, signed in January 1991. He added that the decision to have the deal examined by neutral experts should be interpreted not as an excuse to renege on the agreement but as proof of

Russia's desire to adhere to the regime.

India wants the cryogenic engines for its Geostationary Satellite Launch Vehicle (GSLV) being developed by the Indian Space Research Organization (ISRO) to launch heavy communications satellites. Its 2,350-million-rupee (\$80 million) contract with the Russian space agency Glavkosmos promises to supply it with two cryogenic engines and related technologies that would enable ISRO to build additional engines. The first parts are to be delivered in 1994.

Burbuli's comments have not dispelled India's fears over the fate of the contract. M. R. Rao, chairman of the Indian Space Commission, has admitted that the technology transfer agreement has entered a "difficult" phase. And the prime minister, P.V. Narasima Rao, told parliament last week that "Russia has given all signals of adhering to the contract" but that there remains an element of uncertainty.

Space officials believe that the ISRO-Glavkosmos contract meets all the obligations of contracting parties under MTCR.

The US objections are seen as a move to cripple the civilian GSLV programme and prevent India from becoming a competitor in the launch business.

India has a well-developed space infrastructure and is one of the few countries with a launch centre near the equator — an ideal location for launching geostationary satellites. The GSLV, once developed, will be available for commercial launches of satellites weighing up to 2.5 tonnes.

ISRO scientists admit that the GSLV programme will be delayed if Russia fails to deliver the engines and transfer the technology. An ISRO project to develop cryogenic engines indigenously was abandoned in 1989 when the Russians made their offer.

"If Russia scraps the deal now, we will have to start all over again" says one ISRO scientist. "And we do not know how long it will take to develop a prototype." He said that ISRO has plans for a non-cryogenic stage that would be heavier and more costly than the existing model if the Russian agreement is cancelled.

K. S. Jayaraman

Korea invites West to help it narrow the gap in technology

Seoul. The South Korean government is asking Western and Japanese scientists to help its companies and government research laboratories to catch up with some of the most advanced technologies of the developed world. But Western governments and Japan see little advantage for their scientists in participation, and Korea is now turning to the former Soviet Union for help.

The Highly Advanced National Project, more popularly known as the G-7 project, is intended to put Korea on a par with the G-7 nations (Japan, United States, Canada, France, Italy, Germany and the United Kingdom) by 2000 (see *Nature* 354, 176; 1991). About 120,000 million won (US\$160 million) has been set aside by the South Korean government in the current fiscal year, and the total proposed government investment by 2001 exceeds US\$3,000 million with a matching commitment from Korean industry (see *Nature* 355, 193; 1992). The first projects, to be announced next month, will cover technologies that range from semiconductors and high-definition television to biotechnology and pharmaceuticals.

South Korea's minister of science and technology, Kim Jin Hyun, hopes that by the end of this decade 20 per cent of the funds for the project will go to joint international research. His aim is to turn South Korea into the Switzerland of Asia, with international research teams of many nationalities working in Korea's institutes.

But he says the project's aims have been "misunderstood" by some advanced nations, in particular Japan. The goal is not to catch up with all of the technologies of the advanced world. Rather, South Korea wants to establish a niche in certain technologies so that the nation can survive "between the huge cheap labour market of China, with 1.2 billion people, and Japan, with its highly advanced technology".

The South Korean economy has slowed in recent years following decades of rapid growth. Democratic reforms in the political system have led to increased labour costs, while South Korea's comparatively weak protection of intellectual property rights has made advanced nations wary about licensing technology to South Korea. These factors have driven South Korea's trade balance into deficit after years of surplus, and the government sees development of indigenous high technology as the only solution.

Seven important near-market technologies — semiconductors, high-definition television, integrated services and data networks, electrical vehicles, intelligent computers, pharmaceuticals and computerized manufacturing systems — are targeted in the

G-7 project. In addition, seven areas of more fundamental research will be supported, including biotechnology, new materials and the development of environment-friendly technology.

The South Korean government is seeking the cooperation of scientists in advanced nations. A \$15 million loan from the World Bank will enable it to hire foreign experts as project advisers, says an official at the Korea Institute of Science and Technology (KIST). Government officials also hope that scientists of advanced nations will join some of the research projects. But Western government officials question whether Korea has enough to offer in return for such help.

Many of the areas identified in the programme, such as high-definition television and biotechnology, are "minefields" of problems in intellectual property rights, says one Western science officer in Seoul. Although South Korea has recently reached agreements with the United States and the European Communities over some issues involving intellectual property rights, Korea has yet to demonstrate whether the agreements will work. Korean officials play down the problem, with Chong-Won Lee, assistant minister of science and technology, saying that there is "no reason to worry about intellectual property rights".

South Koreans are particularly riled by Japan's unwillingness to collaborate. Kim and other South Korean government officials approached the Japanese Ministry of International Trade and Industry (MITI) last

year offering to join Japan's Intelligent Manufacturing System (IMS) project, an international project to develop computerized manufacturing systems (see below), one of the targets of G-7. Although Japan is eager to enlist the United States, Europe, Canada, Australia and the nations of the European Free Trade Association, South Korea and other newly industrialized nations have been given the cold shoulder.

The only country showing a keen interest in participation in the G-7 project is the former Soviet Union. The Commonwealth of Independent States (CIS) will send about 70 researchers to KIST this year for stays of a few months, and about 40 will participate in the G-7 project. The CIS researchers will collaborate on research in materials, precision engineering, material processing and aerospace technology, Lee says.

Science officers at Western embassies in Seoul say it is too early to say whether European or US researchers will participate. However, South Korea's top scientists have strong informal links with US universities and industry based on their training in the United States, and there is a large community of Korean scientists and engineers working there. Kim has also described the project to D. Allan Bromley, science adviser to President George Bush.

Within Korea, some 30 government research laboratories at institutes and universities that have recently been designated as centres of excellence will be involved in the G-7 project, as well the newly established Korea Academy of Industrial Technology. But while a few of these new research organizations have excellent facilities and staff with international connections, others do not. And observers are sceptical that Korea has sufficient talent and top-flight facilities to carry out the ambitious goals of the project.

David Swinbanks

Automated factories ahead

Tokyo. After years of delay, the Intelligent Manufacturing System (IMS) project, an international effort proposed by Japan to develop the automated factories of the future, is beginning to move forward.

Following an agreement in February between the United States, Canada, Europe, Australia and Japan at a meeting in Toronto (see *Nature* 355, 755; 1992), an IMS technical committee of industrialists and academics from these nations and regions met in Tokyo last month to initiate a two-year feasibility study. Six research themes were selected: enterprise integration, global manufacturing, system component technologies, clean manufacturing, human and organizational aspects and advanced materials processing.

In the next few weeks, companies and academics in each of the regions will indicate which research themes they are interested in, and in early July the committee will meet in Stuttgart, Germany, to select three or four more-precisely defined research topics. Then international research consortia will be eligible to participate in the first IMS pilot projects, which should be selected by the end of the year, according to Yuki Furukawa of the IMS technical committee.

Some newly industrialized nations have been denied a chance to participate in the feasibility study (see above). Their status will be discussed by the international steering committee of IMS at its meeting in Finland in July, Furukawa says.

D.S.

Big sciences united in reshuffle of SERC

London. A restructuring of the UK Science and Engineering Research Council (SERC) has thrown together nuclear physics and astronomy and given one board the responsibility for most of SERC's big international projects. The change, which will take effect in the autumn of 1993, sets Britain apart from countries such as France, the United States and Germany, where nuclear physics and astronomy are largely managed separately.

Most commentators have greeted the change favourably, saying that the advantages outweigh the possible conflicts of interest that may arise. Although big-science projects will still have to compete for funds, this will take place within the structure of the new board rather than between boards at the council level. It is hoped that the new forum will permit a more informed discussion of the proposed science, the level of funding being requested and issues peculiar to international science.

A more explicit review of the way in which SERC handles international issues will take place under the most recent corporate plan, but details of this review are not yet public. Between now and the implementation of the new board structure, alterations are also expected to be made to the internal organization of each board. It is these changes that will have the greatest effect on researchers applying for grants.

The main aim of the restructuring, which has been on SERC's agenda for some years,

was to reduce the number of interdisciplinary committees that had sprung up at the boundaries between the old order of boards. These were seen by the SERC's management as too bureaucratic and as an indication that the existing board structure did not reflect the state of British research.

Under the new structure, a Physics, Space and Astronomy Board will handle astronomy, astrophysics, Solar System and planetary science, particle physics, nuclear structure physics, atomic physics, plasma physics and Earth observation and geophysics. This arrangement reflects a merging of the old Nuclear Physics Board and the Astronomy and Planetary Science Board, plus some stray physics disciplines from the old Science Board.

A Science and Materials Board will handle materials science and engineering, condensed matter physics, mathematics, chemistry, biology and science-based archaeology. An Engineering and Technology Board has absorbed the old Engineering Commission, covering all the major branches of engineering, and will also handle biotechnology, information technology, computers applied to manufacturing and industrial clean technology.

Although the new structure proposes that materials science and engineering should be handled by the Science and Materials Board, there is apparently still some dispute about how this arrangement will work in practice.

Ian Mundell

Australian universities squeezed

Sydney. Australian universities have reacted angrily to a government proposal to spend more on research by taking the money from a fund used for their general operating costs.

The Ministry of Finance has proposed adding up to A\$160 million (US\$120 million) to the existing A\$280-million federal research grants programme, a suggestion that would normally be greeted with applause. But the catch is that the extra money will be diverted from the general federal grants made annually to each university. Australian universities depend heavily on federal funds, which are used to operate libraries and other university services as well as to repair and renovate buildings.

The universities say that they need more money to ease a growing shortage of space on campus. Diverting their discretionary funds into research grants, they argue, will not help. The universities would still receive

the same amount of money as before, but it could be spent only on specific research grants to faculty.

The proposal was submitted late last month as part of the government's request for next year's budget. A decision will be made in August.

The issue is far from settled. University officials say that they are still feeling the effects of a similar shift two years ago of 1 per cent of their general operating funds into research. John Clark of Macquarie University in Sydney, speaking for the Australian vice-chancellors (university heads) committee, says that some departments are so crowded that new graduate students lack desks and that limited laboratory facilities and crowded libraries are hampering research. Reducing discretionary funds to universities would make this situation worse, he said.

Mark Lawson

Hughes Medical Institute reaches further out

Washington. Later this year, the Howard Hughes Medical Institute will make its second major expansion outside the United States by funding 25 researchers in Britain, Australia and New Zealand.

The five-year awards, along with the continuing grants from last year's forays into Canada and Mexico, will bring the budget of Hughes' new international programmes to nearly \$5 million a year — slightly less than 10 per cent of its annual grants programme, which is separate from the institute's main research effort and its more than 200 investigators. Although the grants programme is a small amount of the institute's total research spending — about \$220 million in 1990 — US tax authorities require Hughes to spend most of its money on scientists who are officially employees of the institute. Because it is difficult to hire foreign scientists as Hughes employees, the international programme is expected to stay a part of the smaller grants component, which began by funding pre-graduate science education and is free of the tax restrictions.

The new programme began to take shape last August, when Hughes officials asked medical school deans in the three countries to nominate researchers. They selected 300 nominees, each of whom was asked to submit proposals. A panel will evaluate the proposals in September, and the winner will be announced by the end of the year.

Hughes officials say their decision to pick three anglophone countries in its first reach across the oceans was based on the fact that Hughes panels find it easier to evaluate research quality in English-speaking countries. Britain also had a boost because of the "feeling that the scientific community owed a great deal to UK advances", says Purnell Choppin, Hughes president. Researchers in the three countries tend to work closely with each other, and the well-publicized funding crisis in British science also "entered into our decision", he says.

Choppin points out that the institute considered several other regional and scientific groupings, at least one of which may be selected in a potential third round of grants in the next year or two. But Hughes has resisted pressure to support research in the former Soviet Union and eastern Europe, in part because of the institute's determined concentration on research quality.

"Everything we do is based on an ability to identify the most promising scientists," says Choppin. Layers of bureaucracy and institutional politics make it difficult to separate the wheat from the chaff in the former Soviet Union, he says.

Christopher Anderson

Foreign contributors hold fate of two SSC detectors

Dallas. US physicists are crisscrossing the world in an unprecedented search for ways to reduce the cost of the two detectors they hope to build for the Superconducting Super Collider (SSC). Their travels to such non-scientific outposts as Albania and Ecuador will test the idea that researchers and industrialists in other countries are eager to share with the United States the cost of discovering the underlying forces of nature.

Until this year, the question of foreign contributions to the SSC has been largely a philosophical debate about whether the United States is capable of attracting significant international support for a major research project. But for the scientists who must build the two massive detectors — known as SDC (Solenoidal Detector Collaboration) and GEM (Gamma-Electron-Muon) — that will carry out the laboratory's experimental programme, the issue has suddenly become very real: if other countries do not pledge nearly half of the \$500 million or so that each detector is expected to cost, that programme will have to be trimmed. It is even possible that only one of the detectors will be built.

"The amount available [from the US Department of Energy] is not sufficient", says George Trilling of Lawrence Berkeley Laboratory and the University of California at Berkeley. "Even with outside help, it's not clear that we will have enough."

With construction workers poised to build the 54-mile-long tunnel that will house the giant machine and with magnet developers increasingly confident that their creations will steer two opposing streams of protons toward their mutual annihilation, attention is turning to the two detectors that will record the actual science to be carried out 250 feet beneath the rolling Texas plains. As tall as an eight-storey building and weighing some 50,000 tons, each detector involves a team of almost 1,000 scientists and will take until 1999 to finish.

Part of the problem is that the required contributions are an order of magnitude beyond any previous collaborations at such accelerator facilities as Fermilab in Chicago and CERN, the European Laboratory for Particle Physics in Geneva. "It's not something that high-energy physicists can decide to do on their own", says Trilling. "They have to work closely with their funding agencies,

and that takes a long time."

But some of the problem is of their own making. The \$8,250 million price for the SSC — a figure that the Department of Energy (DOE) has promised Congress it will not exceed — includes only \$550 million to build the two major detectors. As each will cost that much, some observers believe that the SSC officials made a mistake in deliberately underpricing their research programme.

The typical collaboration features experimental physicists who share an intellectual curiosity, and who may have already worked together. That is the impetus for participation by scientists from Britain, Italy, France, Germany, Russia, China, India, Brazil and more than a dozen other countries. But the quest for financial support has also taken GEM scientists to Albania and its centuries-old tradition of fabricating copper, as well as to Ecuador and its heritage of fine woven goods.

"What we want from the Albanians is pretty straightforward", explains Bill

Worstell of Boston University, whose colleague, Larry Sulak, has visited the country in the hope of striking a deal with the former outcast nation suddenly eager to build ties with the West. "Any competent machine shop would be capable of doing it. But we could buy their copper at internal prices, and the cost of labour would be very low."

Although the component that the Albanians might fabricate — some 940 obelisks of copper sheeting, each 1.5 metres tall and weighing more than a ton — costs only \$11 million by US estimates, GEM hopes that foreign contributions will add up to savings of some \$60 million in the overall cost of the \$150-million hadron calorimeter. Another potential saving could come from enlisting Ecuadorian labour to insert fibre optic cables into tubes that fit into grooves in the copper sheeting. "Our part of the detector is more easily exportable because much of it is relatively low-tech", says Worstell.

Roy Schwitters, director of the SSC laboratory, says that he is confident that other countries will make substantial contributions to the detectors. But the political and financial obstacles are formidable. Japan's decision, for example, is linked to its overall support for the SSC. If it pays for a portion of the accelerator and the magnets, it is likely to make a sizeable contribution to the detectors as well. But a decision not to invest in the machine itself would dim its interest in the detectors. The European teams, on the other hand, are expected to line up behind CERN's plan to build a large hadron collider, leaving little for the SSC.

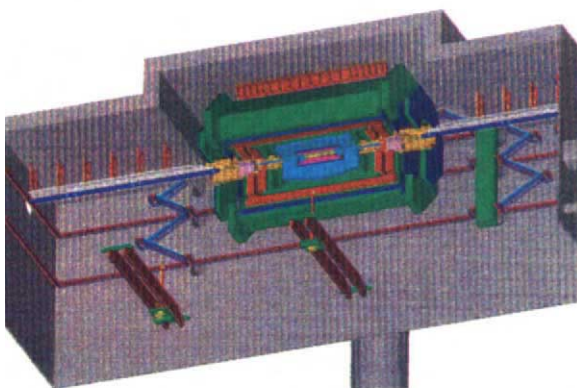
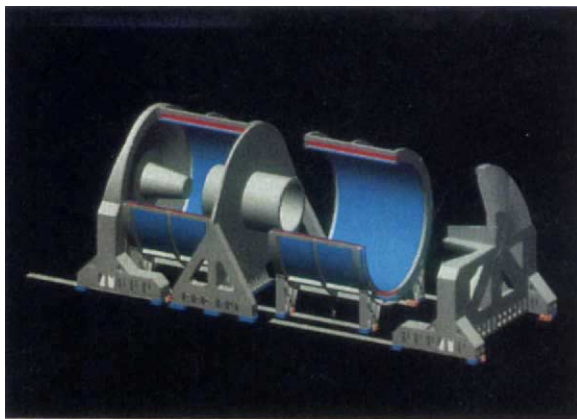
Trilling and other members of both collaborations hope to develop a credible plan about the expected foreign contributors, but they doubt that such decisions will be made as early as DOE officials would like.

"We want to start construction early next year", Trilling says. "But I don't think that we'll have commitments by then."

If the expected support does not materialize, SSC officials will have to face the prospect of doing less science, either by eliminating one or more elements of each detector or moving ahead with only one detector. Collaborators on GEM acknowledge their late start — GEM won approval last summer, while SDC received the go-ahead several months earlier — has put them temporarily behind in the race to nail down foreign contributors.

Schwitters, of course, is not about to reveal his plans. He believes that the laboratory's budget for its experimental programme is adequate, and that international contributions are essential. "We've said what the SSC will cost, and we're not going to budge from that", he says.

Jeffrey Mervis



The GEM (top) and SDC (bottom) detectors require international funding.

Peer review loses out in congressional cutting spree

Washington. A battle between the US Congress and President George Bush over who is responsible for waste in the federal budget has turned into a competition to kill science projects with titles that politicians consider frivolous. The Senate last week passed a bill to kill 31 comically named projects, angering research groups and ignoring the fact that most had passed muster with the scientific community.

In March, Bush sent Congress a list of 246 projects — many of them science oriented — that he deemed wasteful or duplicative. High on the list were projects that Congress had inserted into the budget through a process known as earmarking or 'pork'. Eliminating all the offenders from the 1992 budget would save \$3,600 million, Bush said.

Last week, Congress responded with legislation to save a similar amount. But most of the projects Congress selected for the axe, in separate bills, were those proposed by the administration itself. Whereas the administration singled out such obscure agricultural research projects as "Leafy spurge biocontrol" and "Lowbush blueberry research", Congress simply used the 'silly title rule' that former Senator William Proxmire had followed for more than a decade in choosing his notorious "Golden Fleece Awards".

The bill that the Senate passed directs special derision at the National Science Foundation (NSF) by eliminating projects that the chairman of the Senate Appropriations Committee, Robert Byrd (Democrat, West Virginia), called "executive branch pork".

The projects include social science research on "Holism in psychobiology in the twentieth century" and biological research on "Sexual mimicry of swallowtail butterflies" and "Song production in freely behaving birds". In spite of their comic titles, the projects all passed rigorous scientific peer review, according to Ray Bye, director of legislative and public affairs at NSF.

Research groups object to what they regard as Congress's patent disregard for the scientific process. "We and many others in the scientific community see this as an attack on the peer-review system", says Howard Silver, president of the Consortium of Social Science Associations. "It's the old Proxmire silly-title game carried one step further." Bye believes instead that science became an innocent victim of an election-year feud between Congress and the administration.

A similar bill in the House of Representatives does not strike down any NSF projects, although it would take \$5.8 million in unspecified funding from the National Institutes of Health and \$4 million in research funding from the National Aeronautics and Space Administration.

This week the Senate and the House were expected to meet in conference to reconcile the two versions, and research groups hope that the 'funny-titles' language will be deleted. But even if that attempt fails, the legislation could be vetoed by the president because it reduces funding for such favoured administration programmes as the Strategic Defense Initiative.

Christopher Anderson

Press sues to open White House science meetings

Washington. A publishing group sued the US President's Council of Advisors on Science and Technology (PCAST) last week in an effort to force it to open its meetings to the public.

PCAST, a group of leaders from academic institutions and industry, provides advice to the White House through its Office of Science and Technology Policy. All but a few hours of its two-day monthly meetings are conducted in private.

The council has claimed that its deliberations qualify for exemptions to the US law that requires federal advisory committees to hold their meetings in the open. Arguing that most of PCAST's discussions are neither secret nor sensitive, the lawsuit contends that shielding the meetings from the public is illegal.

Bureau of National Affairs, Inc (BNA), a group that produces several science policy newsletters, initiated the suit. *Nature* and *Science and Government Report*, a biweekly newsletter published in Washington, have filed supporting affidavits and are co-plaintiffs.

The plaintiffs requested a temporary restraining order to halt a two-day meeting of PCAST held last week. At a hearing, Judge Thomas Hogan of the US District Court for the District of Columbia declined to stop the meeting, but ruled that its first day should be opened.

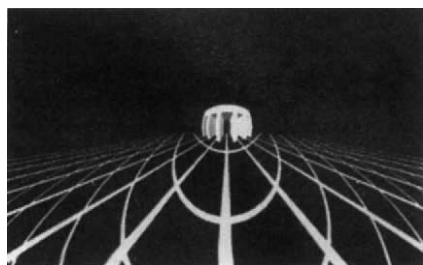
In defending its decision to close most of last week's meeting, PCAST claimed that parts of its discussions should be held in private because they dealt with personnel matters, a report in progress on the health of US universities and final reports on computing and biodiversity. All these topics, PCAST claimed, fall under exemptions to the federal law that requires open meetings. Another discussion on the UN global warming conference to be held next month in Rio de Janeiro needed to be kept confidential to avoid revealing details of the US negotiating strategy, it claimed.

Under the Freedom of Information Act, BNA requested records of previous meetings that had been closed and was able to obtain minutes of almost all the discussion. The fact that the minutes of PCAST meetings are publicly available casts doubt on the claim that the discussions should have been held in private, the plaintiffs argue.

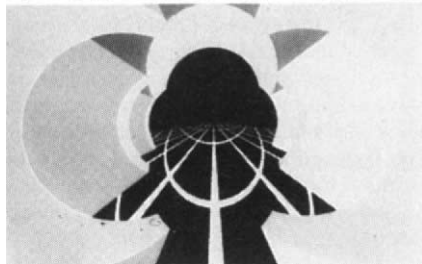
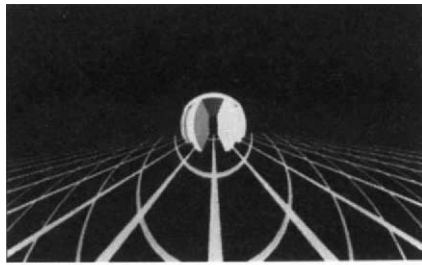
The suit maintains that PCAST also routinely violates provisions that require it to announce its meetings 15 days in advance. Last week, PCAST gave three days notice, citing difficulties in arranging the schedules of participants.

Christopher Anderson

Einstein and Brandenburg



Flying through the Brandenburg gate at 99 per cent of the speed of light. The simulated distortion effect, demonstrating Einstein's Theory of Relativity, is created by one of the many interactive computers available for the amusement and education of visitors to the Deutsche Museum's new astronomy wing, opened last week in Munich.



Austrian system squeezes out young clinical researchers

Munich. Clinical researchers in Austria do not have enough time to meet the requirements for tenure because they are asked to perform too many other academic tasks. That conclusion is based upon a survey of the medical faculty at Innsbruck University and supports long-standing complaints against the country's *habilitation* (tenure) system.

Clinical researchers, who include those working in molecular biology but not necessarily with human subjects, reported that the need to carry out administrative, teaching and patient functions leaves them with an average of only five hours a week in the laboratory. That is not enough time to complete the work needed to generate the number of publications required to move up the academic ladder. Although the Research Minister, Erhard Busek, is sympathetic to the problem, the federal and state governments disagree on the best way to spend what little money is available to relieve some of the pressure on these scientists.

The survey of nearly 450 (60 per cent) of the medical faculty was commissioned two years ago by the Tyrolean Chamber of Physicians in an attempt by Busek to obtain data to support complaints from researchers. Organized by Kurt Grünewald, chairman of the chamber's *Mittelbau* (all faculty except for full professors), it is the first survey in Europe to address the working conditions of untenured research workers.

In Austria, PhDs or medically qualified doctors wishing to take up a university research career must first complete a two-year contract, during which time they must publish a proportion, usually around 50 per cent, of the minimum required for tenure. They can then apply for a five-year contract (or four years in the case of basic biosciences). In these five years they must complete, and in practice usually exceed, a publishing requirement that rewards quality as well as quantity (articles in more important journals are valued more highly than those in lesser-known journals), as well as demonstrating overall expertise in their field.

Researchers get only one chance. Failure at the end of either contract terminates any possibility of a research career.

The survey quantifies the differences in the way in which basic scientists and those in clinical research spend their time. It shows that basic scientists spend more time than clinical researchers on administration and teaching — six and nine hours compared with four and four hours, respectively — and four times as much time in the laboratory — 20 versus 5 hours. The shortfall in research hours in clinical disciplines is made

up at weekends; scientists spend around 10 hours outside their normal working day catching up. But clinical researchers have to spend an average of 33 hours per week on patient care. But even this time competes with overtime clinical work which averages 24 hours a week.

Gerald Zernig, a university assistant who has a hearing this month for tenure in pharmacology at Innsbruck University, has worked in both basic and clinical disciplines. "It surprises me that scientists in clinical disciplines are able to survive and make it under this pressure," he says.

Grünewald says that more staff are needed to do routine patient work and administration, a move that would free tenure-track researchers to spend more time at the laboratory bench. But it is not just a question of money. Whereas most university departments are funded almost exclusively by the federal government, two-thirds of the funding for clinical departments come from the local states, such as Tyrol. "We have two bosses," says Grünewald. "Our work is split between the university and the state clinic. Pressure would be eased if there were a clear separation between the two."

The problem is not unique to Innsbruck. Helmut Tritthart, dean of the medical faculty at Austria's second largest university, in Graz, has long campaigned for greater investment in university clinical research. "In California, clinicians can spend 50 per cent of their time on research." If the situation does not improve, he says, clinical researchers will become a dying breed.

Busek defends the amount of money his government spends on research, pointing out that it has grown by 10 per cent in the past three years. But he says his immediate priority is to expand funding in Vienna, where a new 2,000-bed hospital will open soon. He hopes that the staffing levels in clinical research departments can be increased within the next three or four years. The federal government and the state governments of Tyrol and Steiermark are also negotiating how to divide the bill for clinical research; neither side wants to take on prime responsibility.

Two options remain if the delay is extended. The faculty could report formally to Busek that it is unable to carry out its duties. The ministry would then be obliged to investigate and propose legislative action to reverse the situation. Or the faculty could work to rule, doing their research and treating emergency patients only. Grünewald says that either action would be used only as a last resort because of its impact.

Allison Abbott

IN BRIEF

Las Vegas. American Telephone and Telegraph (AT&T) announced last week that it will end its 43-year role as contractor for the federal government's Sandia National Laboratory in Albuquerque, New Mexico. The decision gives the US Department of Energy 16 months to find another company that is willing to manage the government's primary facility for maintaining its stockpile of nuclear weapons.

AT&T has managed Sandia on a non-profit basis since 1949 following a request by President Harry Truman to help the country defend itself during the early days of the Cold War. Its current five-year contract expires on 30 September 1993.

Both organizations have changed since that time. Once a monopoly providing telephone service nationwide, AT&T is now an international competitor for global markets in communications, computing products and services. Sandia has also taken on a broader role, encompassing research on energy and environmental topics.

Mary Manning

Munich. The 10-day strike by the German public service sector cut sharply into attendance at Analytica, the world's largest laboratory and process instrumentation show held in Munich last week. The challenge of closed airports and intermittent train and subway service resulted in a crowd of only 36,000 at the four-day show.

Foreign visitors were especially inconvenienced by the work stoppages, which ended when the government finally agreed to meet the compromise wage demand, only slightly higher than its original offer, proposed by the unions.

Allison Abbott

Washington. A \$7.5 million funding guarantee from a private foundation will allow the Columbus telescope project to rebound from last year's loss of one of its partners. Peter Strittmatter, director of the University of Arizona's Steward Observatory, which is leading the effort to build the binocular telescope on Arizona's Mount Graham, says that other partners are expected to join. But the guarantee from Research Corporation, an Arizona-based non-profit research foundation, will allow the project to resume immediately. Eventually Research Corporation plans to have about a \$2 million share in the \$40 million project; it plans to distribute the corresponding viewing time to researchers at small universities and others who would otherwise not have access to a state-of-the-art telescope.

Ohio State University, one of the project's three original partners (along with Italy's Arcetri Astrophysical Observatory), pulled out last year, leaving the telescope at least \$15 million short (see *Nature* 353, 97; 1991). But subsequent negotiations (prompted by the threat of a lawsuit) have brought the parties to a reconciliation of sorts.

Christopher Anderson

Greenhouse dilemma

SIR — Lave *et al.*¹ contend that the global climate change research programmes in the United States and Europe “will do little to provide a solid scientific foundation for policy decisions in the next decade”. The “management and focus” of these programmes, they argue, must be restructured and explicitly designed “to answer crucial policy questions” and “to produce answers when needed to policy makers”. Their analysis is flawed as follows.

First, they call for a directed research programme in which tough administrators can discern which projects are “on target” and which are “wasteful”. Implicit in this proposal is the assumption that we understand how the climate system works — that advances in fundamental knowledge (difficult to anticipate, much less produce on demand) are irrelevant. Yet within the past decade an ‘ozone hole’ has appeared over the springtime Antarctic², and anthropogenic aerosols³ and gases other than carbon dioxide⁴ have been recognized as having an effect on climate. These and many other ‘discoveries’ are highly relevant to policy-making and show, moreover, that the inadvertent global change experiment on which humanity has embarked remains largely uncharted territory.

Second, giving top priority to the needs of policy-makers assumes that policy-makers are in a dispassionate position to assess what information they require. The history of global change research, however, demonstrates that it will often produce information that governmental representatives find extremely inconvenient. We submit that the independence of scientific programmes from immediate policy concerns must be strengthened, not eliminated.

Third, it may be true that global change research will fail to provide clear policy guidance within the next decade, but blaming science for this failure and suggesting that tougher management could somehow force science to provide the policy-relevant answers displays a dangerously simplistic view of both the global climate and the way in which basic research influences public policy. It was free inquiry, not policy-orientated research, that uncovered the global warming problem in the first place (for example, refs 5 and 6) and that has led to every major advance in understanding since. These advances include the discovery of inherent complexities in the climate system (for example, feedbacks of unknown sign involving clouds, ocean currents and biota) — news that may be frustrating but is nevertheless important to policy-making.

Finally, Lave *et al.* assert that the

agenda of research should not be “simply to advance knowledge” — a strange enough statement in a journal devoted to seeking scientific truth. Let’s take note, however, that the pressing need to anticipate climate change is also an unprecedented opportunity to conduct Earth-systems research on the scale such research demands. Were this project to become a directed research effort, laid out in advance and held to a strict set of topics and timetables, the result would be certain disaster for basic science and a far greater likelihood of unexpected calamity for the Earth’s inhabitants.

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Coelacanth tissue bank

SIR — Coelacanths (*Latimeria chalumnae*) are accidental yet inevitable victims of the oilfish fishery on which depends the economy of the Islamic Republic of the Comores in the western Indian Ocean. Coelacanths die within a few hours of capture, and the impossibility of preventing coelacanth catches, as well as regulating trade in those that are caught, makes the long-term preservation of such material as is available imperative.

A feasible and effective way to do this would be a long-term programme of deep-freezing coelacanth material, so that it might be available for future generations of researchers to examine. To this end, the Max Planck Society has instituted just such a scheme.

In August 1991, a gravid female containing 26 late-term fetuses was caught off Mozambique — the only occasion, apart from the very first catch in 1938, on which any coelacanth has been caught outside the Comoro Islands. The specimen was given to Dr Cabral of the Maputo Natural History Museum in Mozambique, who donated frozen

fetuses to the J. L. B. Smith Institute of Ichthyology in Grahamstown, South Africa, and the Max-Planck-Institut für Verhaltensphysiologie in Seewiesen, Germany. The Max-Planck specimens joined those of an adult and a sub-adult fish already deep frozen at -80°C . Tissue from this bank is available for scientific study on request, and interested researchers should address their enquiries to me.

Hans Fricke

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National pride

SIR — Your lamentation of crumbling empires and apprehension of emerging nation states (*Nature* **355**, 659; 1992) is misplaced. That is the way of nature. The one overwhelming process that keeps life going is evolution, and you can’t have evolution without competition. The more competitors, the better the chance of hitting the jackpot, so Mother Nature wants more diversity: more cultures, more languages, more nations.

Language differences among nations perform a very useful function as filters of information. By letting in desired information and excluding the rest, a nation can develop a very distinctive way of life, evolve in its own way. Thus, the more nations, the more ways of life, the better the chance that one of them will make an unprecedented leap of progress.

Perhaps the biblical fable of Babel has more truth in it than previously supposed, although in a perverse manner.

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Rajiv Gandhi

SIR — A recent News article (*Nature* **356**, 185; 1992) about cooperation between Russia and India says that the former Indian leader, Rajiv Gandhi, was assassinated and thus removed from office. But in reality, he lost office when his Congress party was defeated in a general election. He had already been out of power for more than a year when he was assassinated.

N. P. Raju

c/o Prof. Dr. E. Gmellin, Max-Planck-Institut für Festkörperforschung, Heisenbergstrasse 1, 7000 Stuttgart 80, Germany

Reality of sequencing costs

SIR — In their interesting article on the *Caenorhabditis elegans* genome sequencing project, J. Sulston *et al.* (*Nature* **356**, 37–41; 1992) anticipate "... the cost, currently estimated at \$1 per base with current methods. ..." of their strategy. How did they arrive at this estimate, and what is included in estimating the cost?

Let me do the following calculation for such a project in Germany. Two fluorescence sequencers (paid off over 5 years) cost about DM80,000 per year, 600 primers synthesized (length 15, DM5–8 per base) is more than DM45,000 for 120,000 base pairs (DM0.375 per bp). DNA preparations, chemicals, enzymes, gels and so on come to at least DM15,000 for 1,500 clones (DM0.12 per bp). If, say, one-third of the authors' time was spent on laboratory work such as cloning, DNA preparation, sequencing and editing (DM75,000 × 19/3) the cost for salaries would be DM475,000 per year. Rent and running cost for a laboratory of 120 people will be about DM30,000 per year. There will be additional costs for laboratory equipment, repair, laser-tube replacement, break-down time, overheads and so on, often neglected by scientists.

With such rather optimistic numbers I arrive at minimal costs of DM645,000 for 120,000 bp per year, or \$3.00 per bp of final sequence. For 800,000 bp per year with full-time employment and 20 per cent overhead, the costs triple to DM2 million and will be close to \$2.00 per bp.

I founded a sequencing company in 1990, in which about 600,000 bases were read for assembly during 6 months from two cosmids by using direct-blotting electrophoresis and colorimetric detection of digoxigenin. The actual costs were close to the above estimate. The costs do not include profits and the work was done by dedicated people. This is in accord with the present funding of the yeast project — requiring very high quality for the sequence data — by the European Commission with 2 ECU per bp, which is close to \$3 per bp.

But it still appears to be a very long way before costs of \$1 or \$0.50 per base pair will be a reality. One should be very careful with such estimates, especially in a scientific paper, because they may have far-reaching consequences for biological research.

Fritz Pohl

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SULSTON REPLIES — I agree with Pohl's essential point that estimates of DNA sequencing costs must be all inclusive and made with care. Unfortunately, in

an effort to shorten the paper and in response to a referee's opinion that cost issues would not be of broad interest, we removed the detailed discussion that was in our original manuscript.

A cost of \$1 per base pair of finished sequence is the current production cost, not the actual cost of sequencing the three cosmids; the latter was indeed higher because of time spent in development. We have now settled down to a production routine, and are able to estimate our actual costs reasonably confidently. In so doing, we have allowed for all the hidden extras (such as fringe benefits, supervision and replacement of minor equipment) that, as Pohl points out, are so easily overlooked, and a 50 per cent overhead for rent and running costs of the laboratory space.

The discrepancy between our estimate and that of Pohl is due to: (1) heavier use of the ABI 373A — only two machines per megabase of finished sequencer per annum; (2) in-house synthesis of oligonucleotide primers, of which only 100 are now made per cosmid; and (3) efficient use of staff by job specialization, with appropriate levels of training for each operation.

The pace of sequencing is sustainable, because the members of the groups, though certainly dedicated and flexible in their activities, are not called upon to work excessively long hours for routine production. (Development work, and writing scientific papers, is another matter.)

John Sulston

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Law of mass action

SIR — John Maddox (*Nature* **355**, 201; 1992) scolds molecular biologists for their naive reliance on qualitative observations, and calls for a resurrection of the law of mass action to explore crucial quantitative features of molecular regulatory networks.

We agree with the doctor's diagnosis and remedy, but would like to point out that the patient's plight is not as serious as might be inferred. In addition to the "army of people called molecular biologists" who uncover the qualitative structure of molecular control mechanisms, there are squads of theoretical biologists who wield the law of mass action to explore the quantitative implications of these mechanisms. As a small selection of recent studies that illustrate the usefulness of this approach, we draw your attention to models of cyclic AMP meta-

bolism in slime moulds¹, molecular events in neurotransmitter release², quantitative triggers of cell division³, genetic switching in early embryos⁴ and interleukin-2 binding to T lymphocytes⁵.

Modern science proceeds largely through the efforts of specialists, so that one cannot expect molecular biologists to deviate much from their spectacularly successful efforts. Other excellent biologists study integrative physiology, and a small group of theorists, from biology and other disciplines, labour to create sound and predictive connections between molecular and physiological levels. Communication among these specialists is essential. *Nature* could play a helpful role here by devoting more attention to existing quantitative and integrative research in molecular and cellular biology.

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Artists' offspring

SIR — We read with interest the recent letter from R. A. Beck (*Nature* **356**, 189; 1992) suggesting the possibility that artists have more sons than daughters. We became sceptical after calculating that among the sample of artists' offspring 52.8 per cent were sons (1,834 of 3,474), compared to 51.9 per cent sons (2,046 of 4,002) among the sample of children of the general population in *Who's Who*, a very small difference indeed. A quick attempt to confirm the chi-square statistic produced a value of 2.072 (not using the Yates' continuity correction), corresponding to a two-sided *P*-value of 0.150. Thus an amusing hypothesis (associated, unfortunately, with a non-trivial amount of data compilation) must be put to rest. If one were to repeat this exercise with five other randomly chosen occupations, one would produce at least one chi-square value greater than 2.072, purely by chance, with probability 0.556.

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More on Gallo and Popovic

The report of the US National Institutes of Health is not so much a finding as a series of findings on the research that led to the discovery of the AIDS virus.

ROBERT C. Gallo may have been cleared of misconduct charges in last week's report from the US National Institutes of Health (NIH) Office of Scientific Integrity (OSI), but the report is so equivocal that he is unlikely to regard it as the most glowing testimonial he has ever received. It is not surprising that Gallo's lawyers are ready with a rebuttal of the criticisms of their client with which the report is spattered.

So too are the lawyers for Mikulas Popovic, Gallo's principal collaborator in 1983 and 1984, who is accused of actual misconduct (see *Nature* 57, 3-4; 7 May 1992).

The document is nevertheless not so much a finding as a series of findings, on issues that are successively important and relatively much less so. The authorship of the report, labelled "proposed final investigative report" in recognition of the supervisory role of the Department of Health and Human Services, is not directly stated. The document now in circulation deals separately with the two phases of the two-year investigation of Gallo's laboratory — the "inquiry" launched in February 1990 and the "investigation" launched in October of that year, in the light of the report of the inquiry.

The OSI inquiry team is listed as Dr Suzanne Hadley (forced out of OSI last summer, who is described as an "expert in research psychology"), Dr Jules Hallum (director of OSI), Dr Paul Parkman (ex-director of the Food and Drug Administration's research and evaluation centre) and Dr Edmund Tramont from the US Army research institute.

The extramural members of the investigation team were Professor Kenneth M. Berns of Cornell University, Professor Priscilla A. Schaeffer of the Harvard Medical School (and the Dana-Farber Institute) and Dr Michael McGrath from the University of California. After Hadley's departure, her successor Dr Clyde A. Watkins replaced her on the team, which was also assisted by Drs Barbara R. Williams and Pamela J. Baker, also of OSI. References in what follows to "the report" are to the "proposed final investigative report"; the "inquiry" and the "investigation" are thus distinguished.

The report deals with events at Gallo's laboratory from the early part of 1983, when the first report of Luc Montagnier's isolation of the virus now known as human immunodeficiency virus (HIV) appeared in the literature (Barre-Sinoussi *et al.*

Science 220, 868; 1983) to the early part of 1985, when the publication of complete nucleotide sequences of the French virus (then called LAV, for lymphadenopathy virus) and Gallo's version revealed a surprising similarity.

The authors acknowledge the degree to which the inquiry was stimulated by the appearance, in November 1989, of an article in the *Chicago Tribune* by journalist John Crewdson, alleging that Gallo's laboratory had misappropriated the French virus as its own.

Much of the report centres on the circumstances leading to the publication, in May 1984, of a group of four articles by the Gallo group which, according to OSI, "enabled the ELISA-based test for HIV" (*Science* 224, 497-500, 500-503, 503-505 & 506-508; 1984). Gallo and Mikulas Popovic, his principal collaborator at the time, were the only two authors common to each of the four articles.

OSI explains that the first of the articles, entitled "Detection, isolation and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS", whose principal author was Popovic, caused "particular concern", especially because laboratory notebooks requisitioned by the inquiry seemed not to contain data to support the claim that virus cultures had been sustained for five months.

The decision to launch a formal investigation of the evidence supporting this paper is said to have been taken after a meeting with a panel of consultants in September 1990, at which it was also agreed that the evidence for the claims in the three remaining papers was "generally adequate", despite particular discrepancies and contradictions.

In retrospect, this meeting seems to account for the confusion following the announcement in October 1990 by then-acting director of NIH, William Raub, that Gallo had been cleared of misconduct, but that a formal investigation would continue. Those concerned had, for example, concluded that allegations that Gallo had failed to culture virus other than LAV were unfounded, but that the continuing investigation must include Gallo because of his role as laboratory chief.

The inquiry says that it requisitioned virus isolates and other samples accumulated at Gallo's laboratory in the period 1983-84. OSI has more recently commissioned commercial molecular biology laboratories to produce nucleotide sequences of the virus they contain. OSI says that the present version of the report may be amended when these data are available. The document itself contains no explanation of the decision not to wait for the data before transferring the case to the Department of Health and Human Services.

Noting that LAV and HTLV-III_B (the name given to Gallo's virus in 1984) are "remarkably alike", the report says that there are two interpretations of the similarity: either Gallo "knowingly misappropriated the French virus" or that he "unknowingly used and reported it as his own isolate, following LAV contamination" of his own cultures.

Gallo is said to have acknowledged the possibility of contamination to the inquiry, and to have stated that the existence of a number of other isolates of virus in his laboratory "would prove he had no motive to misappropriate the French virus". Data on the nucleotide sequences of the French and US viruses published in February and May 1991 have since been taken to imply that there was contamination at both laboratories — at Bethesda by LAV, in Paris of the isolate known as BRU and by one called LAI.

OSI takes the view that the more recent data "do not speak directly" to the issue of misappropriation. The history of the isolation of HIV at Gallo's laboratory is nevertheless relevant, and central to the accuracy of the article by Popovic *et al.*

First, the inquiry concludes that the origin of the name HTLV-III_B, and its distinction from an isolate called HTLV-III_A, is unclear. What is clear, however, is that both emerged from a procedure "of dubious scientific rigor" in which samples from a total of ten different patients were pooled, and used to infect laboratory strains of T lymphocytes, notably the cell line called HUT-78.

OSI notes that Popovic's notebook was a "loosely related set of pages" in which entries had been made several days after the experiments they described, and that it may have been "assembled" in preparation for the French patent challenge in 1986 and the later OSI inquiry in 1990.

The first record of the inoculation of HUT-78 cells with pooled virus refers to 15 November 1983; OSI says that the statement in the published paper that the samples were "first" shown to be secreting reverse transcriptase is contradicted by the evidence of the notebooks that only one of the three

was tested. The cells were reinoculated with the same three samples a week later and, on 2 January 1984, with samples from a further seven patients. Gallo and Popovic differed in their evidence to OSI about the reasons for the pooling procedure, but Gallo is also reported to have said that "the pooling was done without his knowledge". OSI records that, after May 1984, both Gallo's and Montagnier's laboratories worked on the comparison of LAV and HTLV-III_B, using antigenic and restriction enzyme procedures. Two joint manuscripts were prepared but never published, on the grounds that they would quickly be superseded by the nucleotide sequences that eventually appeared in 1985.

On the question of whether Gallo's laboratory had isolated other strains of virus during the later months of 1983, OSI is nevertheless unequivocal. The articles published in 1984 claimed that virus had by then been detected in or isolated from 48 patients. OSI now says that it has been able to verify these claims for a random third of a list of 32 isolates/detection listed by Gallo for the investigation.

OSI also gives Gallo's laboratory a clean bill of health on the handling of particular isolates cited in the 1984 articles, despite discrepancies noted by Crewdson. These vary from the inversion of the initials of one patient (so that TW became WT) to the early date (in February 1983) of an electron-micrograph published in 1986 (*Nature* 320, 535; 1986), which OSI confirms to be the date at which the sample was received at the laboratory.

On the work at Gallo's laboratory with LAV, OSI says that, "by contrast . . . the records are very sketchy" [compared with those for domestic isolates], and that "responsibility for the poor quality" of the records rests with Popovic. OSI guesses that a partial explanation is that there was "considerable skepticism" in the Gallo laboratory about the Pasteur findings, and that Gallo had explained his interest in LAV as "something [we] are doing for Dr Montagnier".

The records show that Gallo's laboratory received five samples of LAV between April and September 1983, the first of which consisted only of DNA (pro-virus). OSI says that the "ambiguities" about the use of LAV at the Gallo laboratory are greatest for the samples received in July 1983, now celebrated as those left in Gallo's domestic refrigerator.

Although there are no records of Popovic's use of these samples to infect umbilical cord blood, OSI says that LAV cultures were alive and producing virus "well into September 1983". There is a conflict between Popovic's statement (in a memorandum in November 1985) that "we did not have success in transmitting this virus to cells" and his reference to "consistently negative results". The inquiry found that "these statements are not correct".

LAV was also used to infect cord-blood cultures later in September and, after the arrival of a second shipment of samples from the Pasteur Institute, in November. At that time, attempts (two of which appear to have been successful) were also made to infect permanent T-cell lines, two of which were still alive in November 1983. OSI says there are no records of the attempts in January and February the following year to repeat these experiments, although three samples sent for electron micrography were reported negative for virus particles. OSI notes that Popovic was ordered by Gallo to abandon LAV to concentrate on the laboratory's own isolates.

The investigation's more detailed analysis of the disputed paper by Popovic *et al.* inevitably reaches more specific conclusions.

■ The investigation concludes that the claim in the published paper that the culture was "continuous" even though it had been reinoculated on at least two occasions was not a misrepresentation. But one of the expert advisers strongly dissented, on the grounds that "a responsible author writes for the entire audience, not just the careful reader".

■ The statement in the paper that virus had been cultured for "over 5 months", acknowledged to be incorrect when the paper was submitted for publication four and a half months after the first successful culture, may have been the result of an honest error or of the haste with which the paper was prepared, but whether it constitutes "scientific misconduct must be assessed in the context of the totality of the evidence".

■ The investigation considers that quoted estimates of doubling time and of the viability of cells in culture, for which there are no laboratory records and which might be considered to "have been rendered meaningless" by the reinoculation of the culture, do not amount to scientific misconduct but are, rather, "another example of lack of attention to detail which resulted in a false representation".

■ Although samples used in the estimation of reverse transcriptase activity were prepared by two different methods (precipitation with polyethylene glycol and sucrose gradient centrifugation), and might well yield results that are not comparable, a figure showing the reverse transcriptase of several isolates did not distinguish between the different treatments, which cannot be reconstructed from laboratory records. The investigation concludes that the "lack of scientific rigor" is a matter of concern, but that it does not constitute misconduct.

■ Allegations that data for another figure in the disputed paper (Fig. 2a) were deliberately selected to show wide variations of reverse transcriptase activity cannot now be substantiated for lack of the laboratory records.

■ Although "the reader should have been informed" that the data used to generate these two disputed figures were obtained with different viruses, the failure to do so was not a "significant misrepresentation".

■ The claim in the published paper that virus samples had "first" been tested for the presence of reverse transcriptase is judged to be "not true", and the investigation also concludes that the addition of this sentence to the final draft of the paper "suggests that the misrepresentation was deliberate", that its purpose was to "increase the apparent rigor" of the culture procedures and that "this reckless disregard for truth in science" constitutes scientific misconduct. The investigation says it has not been able to tell which of the authors added the misleading sentence.

■ On the statement in the paper that there was evidence to suggest that LAV and HTLV-III are different, but that LAV was insufficiently characterized for a firm conclusion "because [LAV] has not been transmitted to a permanently growing cell line", the investigation reaches a different conclusion. It has tracked the eight successive drafts of the paper, concluding that the statement that "LAV has not yet been transmitted to a permanently growing cell line" made its appearance only in the final published version. Gallo has not denied authorship, and takes responsibility for this section of the paper. In his evidence to the investigation, Gallo said that the evidence to which he had referred of the differences between LAV and HTLV-III was that (reported from Paris) that LAV appeared less frequently to be associated with AIDS than HTLV-III had been found to be, and that he "thought LAV would probably have a higher association with AIDS once better reagents were available". He also told the investigation that it would have "been making fun" of the Paris group to have reported success in growing LAV when that had not been done in Paris. The investigation notes that the assertion that LAV had not been grown in culture might be regarded as "self-serving", but says that the plan for US-French collaboration on the viruses, the apparent differences between LAV and HTLV-III and the terms on which LAV had been supplied to Gallo's laboratory persuaded it that no misconduct had been involved.

■ One of the more arcane complaints against the published paper is that the abbreviation N.D., defined in the legend of a table as "not done", was understood by Popovic to be "not done at the time of writing the paper", although the data had come into being soon afterwards and although three of the four measurements concerned proved negative (against the drift of the argument). The investigation concludes that "treating data in this manner was misleading" and that it constitutes misconduct.

■ On a question formulated by the inquiry ►

about the validity of an entry in a published table that showed a 10 per cent immunofluorescence assay of a cultured clone, whereas the relevant notebook included the legend "very few cells positive", the investigation does not accept Popovic's claim that he had read the data separately from his colleague, and that the published data were averages of the two datasets. With a trace of irony, the report says that "he noted that he unfortunately could not adduce written documentation", and concludes that the treatment of this one datum constitutes misconduct.

■ On three allegations about a table representing data gathered in an investigation of the reactions of individual patients to particular assays, the investigation rejects two, but says that the legend N.D. was used to conceal awkwardly negative results, and thus constitutes misconduct.

■ One of the most contentious issues in the report is the origin of the T-cell line called HUT-78, now acknowledged to have been established by Dr Adi Gazdar. The earlier inquiry had concluded that Gazdar had not been given credit for his work, and that the origin of the cells had been obscured by a change of nomenclature and by the incorrect description of the line as having originated in a patient with lymphoid leukaemia. A related issue is whether a letter in the *The Lancet* in December 1984 with Gallo and Popovic as two of three authors misrepresented three cell lines as different when, in reality, they were all derived from HUT-78.

The investigation says that the issue became more important as its work went on, chiefly because of the confusion about the origin of the cells. The report says "it is astonishing" that, in the letter in *The Lancet*, "Dr Popovic used and reported on a cell line which he still has not been able to identify with certainty".

The investigation was plainly offended by the conflict of the evidence from Popovic and Gallo that "they could not identify the source of the HT cell line any more precisely than they had reported" when early drafts of the disputed paper, including one in Popovic's handwriting, "fully and accurately identified the source as a patient with Sezary Syndrome".

The investigation is also critical of Popovic for having submitted the *Lancet* letter referring to three supposedly distinct cell lines when he "knew or should have known" of their common origin.

On at least two occasions, the investigation reports that Popovic was instructed by Gallo to investigate the origin of the cell line; the investigation complains that he was "negligent in failing to ensure the accurate conduct and reporting of research". The investigation says its members did not arrive at a consensus on the question whether the objective was to disguise the origin of the cell line in which HIV was eventually grown. The investigation concludes with a listing of 20 instances of "knowledgeable

Gallo and Popovic lawyers reply

In letters to Bernadine Healy, director of the US National Institutes of Health (NIH), attorneys for Robert C. Gallo and Mikulas Popovic take exception to certain conclusions in the draft "final" report from the NIH Office of Scientific Integrity (OSI).

Joseph N. Onek, who represents Gallo, takes issue, for instance, with the suggestion that "the results of the sequencing [of virus samples from 1983 and 1984] will shed additional light" on the allegation that the French virus was "misappropriated". Much of the sequencing is complete already.

In a letter dated 11 February 1992, Onek writes: "What is the possible basis for the belief that the results of the sequencing will shed additional light on the matter? This is truly a self-serving statement by the OSI to justify its expensive sequencing efforts and to avoid the obvious conclusion that any contamination was accidental. The statement is pernicious because it leaves open an issue which should be closed."

Onek also challenges OSI's conclusions about the origins of the HUT-78 cell line which Popovic cloned to obtain a cell line he called H9. In the conclusion of its report, OSI claims that Gallo "misrepresented" the origins of the H9 cell line. Onek says: "This charge is also inconsistent with the body of the report. The report never states that Dr Gallo misrepresented the origins of H9 Moreover, he and Dr Popovic made the H9 cell line available to researchers throughout the world and Dr Popovic published the HLA phenotype of H9 in 1985 Despite these facts, and despite the extensive efforts made by Dr Gallo and Dr Popovic to find the origins of H9, the reports suggests that Dr Gallo should have done still more. The OSI seems unaware that millions of people are dying of AIDS and that both Dr Gallo and Dr Popovic had higher priorities Dr Gallo never claimed to have developed H9 himself and gained nothing by the confusion as to its origins."

Popovic is represented by attorney Barbara F. Mishkin who, like Onek, challenges the OSI's handling of the allegation of misappropriation. In a letter to NIH director Healy dated 13 February 1992, Mishkin writes: "We specifically request that OSI address, and lay firmly to rest, suggestions . . . that our client stole, or misappropriated, the French virus."

"Inasmuch as the sequencing report demonstrates that every identifiable virus that our client put into his 'pool' was a novel virus (that is, neither LAV nor LAI), it is clear that he did not 'use' the French virus to establish his continuous culture. That Popovic's pooled culture and the Institut Pasteur's LAV/BRU were both subsequently contaminated by LAI was unknown at the time the *Science* papers were published and refutes assertions of intentional misappropriation. In all fairness, NIH should make this clear."

"It is equally important to emphasize that research on the cutting edge cannot always be as precise and carefully planned as one would like. Despite the trivial and unintended inaccuracies, the central finding of Popovic *et al.* remains valid and his reagents (virus-expressing cell lines) are widely used even today."

The OSI report is now in the hands of James Mason, the assistant secretary for health in the Department of Health and Human Services, of which NIH is a part. It is now up to Mason to issue a final opinion after evaluating the report and the rebuttals.

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misreporting or errors" which, it says, is an inordinate number for a paper only three and a half pages long. Acknowledging that some are merely drafting errors, it says that eight constitute scientific misconduct. "Taken together", the report says, "these errors represent evidence of a level of haste and error that cannot be condoned." The report is also critical of the editorial handling of the four manuscripts, saying that "many of the errors of ambiguity and inconsistency" should have been discovered before publication.

Plainly the investigation does not wholeheartedly follow the authors' defence that a blood-test was necessary for screening blood used in the treatment of haemophiliacs, that delay in publication might hazard the chance of securing patent protection and that there was in any case political pressure to publish quickly from the Department of Health and

Human Services." The investigative team was not convinced that the speed of preparation, whatever the reason, made the knowing misrepresentations inevitable or excusable.

The investigation's recommendations on Gallo are that he should be held directly responsible for four minor discrepancies out of the total 20, and that "he breached his overall responsibility . . . to ensure the accuracy of the paper", but that this does not constitute misconduct. The report says that the outside advisers were split two to one on this recommendation, with the odd person holding that "Dr Gallo's negligent conduct . . . coupled with his apparent disregard in this instance for accuracy and responsibility in the conduct and reporting of scientific research, did constitute misconduct".

John Maddox

A fine time for contractual alterations

Andrew Huxley

IT has long been supposed that the elementary event which produces tension in a muscle fibre, or causes it to shorten, may be a change in the orientation of the head of a myosin molecule; the head spans the gap between a thick (myosin) and a thin (actin) filament, and acts as a crank pulling the thin filament into the array of thick filaments which forms the A band of the striation pattern^{1,2}. Such an event would alter the distribution of mass projected onto the fibre axis. In their report on page 156 of this issue³, describing experiments with low-angle X-ray diffraction, Irving *et al.* show that the expected change does indeed accompany the 'working stroke' of the force generators.

Reflection

A feature of the low-angle X-ray diffraction pattern of striated muscle is a reflection on the meridian at a position corresponding to an axial spacing of 14.5 nm, the interval along each thick filament between successive 'crowns' where the heads of myosin molecules project from the backbone of the filament. Nearly a decade ago, H. E. Huxley and his colleagues⁴ showed that the intensity of this spot fell to about half when an active muscle shortened quickly by 0.5–1 per cent of its length, and recovered with a time constant of about 10 ms (frog muscle at 0°C). The time resolution of their measurements (X-ray quanta counted in 1 ms bins and shortening complete in about 1 ms) was impressive at the time. But it was not good enough to show clearly whether the drop in intensity of the 14.5 nm meridional reflection was an accompaniment of the shortening itself or of the redevelopment of tension that occurs in the subsequent 1–2 ms, and represents (in at least one sense) the working stroke of the crossbridges^{5,6}. Irving *et al.* have taken advantage of the improved time resolution (0.2 ms bins) now available at the SERC synchrotron facility at Daresbury to repeat the experiment. They show decisively that the change does not begin until the shortening is complete and that it develops with a time course similar to that of the tension redevelopment (that is, of the working stroke).

In addition to its theoretical significance, the experiment of Irving *et al.* was a technical *tour de force*. The tissue used was a single muscle fibre (from a frog); the step change in length was complete in 0.12 ms; and the length change in the segment of the fibre traversed by the

X-ray beam was recorded continuously with an optical device⁷ which monitors the movement of the striations at each end of the segment with time resolution of a few microseconds.

Irving *et al.* show, by computer modelling, that the intensity change can be well explained by tilting of the myosin heads from an attitude roughly perpendicular to the fibre axis with time course and extent expected from a kinetic scheme previously devised to fit the mechanical responses to step changes of length⁸. They admit, however, that this is not the only type of crossbridge movement that might be causing the X-ray change; it might, for example, be a sliding movement of some of the heads along the thin filament, or it might be attachment at intermediate positions of myosin heads that were not attached to actin before the step. But the observation does appear to be an unambiguous demonstration that the working stroke is accompanied by a redistribution along the fibre axis of the material composing the crossbridges (the heads of myosin molecules).

The experiment is also relevant to the interpretation of a phenomenon reported a few months ago by Lombardi *et al.*⁹, one of the groups responsible for the new X-ray results. It was already known⁶ that when two step releases are applied within 1–2 ms, the subsequent time course of tension is much the same as that following a single release with amplitude equal to their sum. Lombardi *et al.* showed however that when the interval is increased to 10–15 ms, the second release is followed by a quick redevelopment of tension almost as great as if the first release had not taken place. The time in which this 'repriming' of the ability to perform a working stroke occurs is much too short for one to be able to assume that crossbridges have completed their biochemical cycle using one molecule of ATP, which implies that the muscle fibre can perform several working strokes with the energy provided by the hydrolysis of a single molecule of ATP. The time course of the repriming is similar to that of the recovery of intensity of the 14.5 nm meridional reflection that was seen both by H. E. Huxley *et al.*⁴ and by Irving *et al.*³, suggesting that the crossbridges have returned to their previous attitude.

Both the repriming and the recovery of the 14.5 nm intensity, with the observed time course, are predicted by the same kinetic scheme⁸ that accounted

for the initial drop of intensity. The relevant feature of this scheme is that a crossbridge which has not completed all the steps of its working stroke, and which is put into compression by the imposed quick shortening, is able to detach with a rate constant corresponding to the speed of repriming. It can then rapidly reattach further along the actin filament in the attitude corresponding to the start of a fresh working stroke.

Drawback

The drawback of the kinetic scheme as it stands is that it does not set a limit to the number of times that a partial working stroke can be performed with the utilization of one molecule of ATP; in its present form it is therefore thermodynamically inconsistent. One hopes however that a modification to the scheme (for example, perhaps postulating that the crossbridge does not detach but is brought back to its initial attitude by a shortening of the S-2 segment of the myosin molecule driven by the phosphate-release step of the ATPase cycle) may make it satisfactory in that respect without losing its relevant kinetic features.

There have been other suggestions that each crossbridge may perform several working strokes for the use of one molecule of ATP (see, for example, refs 10–12). These are still controversial, and not all of them assume the same timescale as the experiment of Lombardi *et al.*⁹. This is a lively field of research at present, with several groups studying the mechanical behaviour of isolated filaments in preparations where the force contributed by individual molecules can be detected either as noise or as individual impulses. Another technique now in use is quick freezing for electron microscopy; this is probably the only approach that can decide whether the structural alteration in a crossbridge really is a change of its angle relative to the fibre axis. □

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EARTHQUAKES

Three's a crowd in California

Andrew Michael

A TRIO of strong earthquakes near the Mendocino triple junction off the coast of northern California last month gave earth scientists an unusual opportunity to study the tectonics of an area where three, not two, plates meet. Earthquakes are frequent in this area (there were magnitude 7 and 6 events in 1923 and last year, respectively), but the current sequence (with magnitudes 6.9, 6.2 and 6.5) is the largest and best instrumented this close to the triple junction. Beyond the implications for plate tectonics, these events also displayed unusual source properties and produced one of the high-

'great' earthquake around the end of the seventeenth century. Running west of the triple junction is the Mendocino transform fault where the Pacific and Gorda plates slide past one another (see figure).

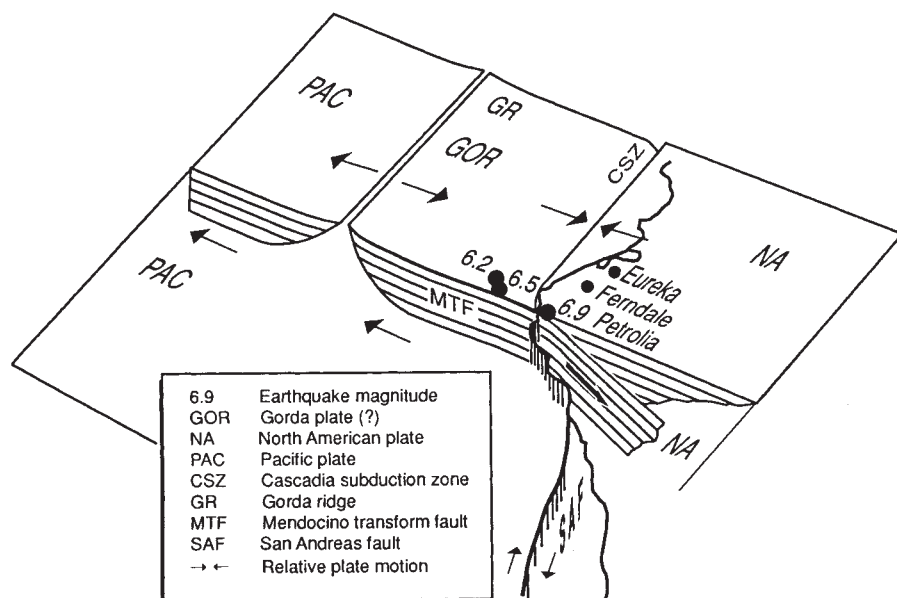
In theory a triple junction is a point surrounded by three rigid plates². However, the seismicity and geology of the Mendocino triple junction reveal a broad zone of deformation and the San Andreas fault and Cascadia subduction zone appear to curve into the Mendocino transform fault over tens of kilometres. This deformation covers

either northwest-southeast or northeast-southwest. The latter orientation would make the earthquake similar to the $M=6.9$ event that occurred off Eureka in 1980, but the former is suggested by the current aftershock locations. Either could be the result of some north-south compression of the Gorda plate which could also cause the rotation of the subduction zone. The source of this compression may be northward motion of the Pacific plate into the Mendocino transform fault due to slip on the San Andreas fault (and resisted by the locked subduction zone) or a mismatch between the spreading direction on the Gorda Ridge and the orientation of the transform fault³.

If the mainshock was indeed on the Cascadia subduction zone main thrust plane, this event is the first known to rupture this increasingly feared fault. The geological evidence, of rapid coastal subsidence and tsunamis along the coast from California to Washington, indicates there have been five events of $M \geq 8$ in the past 1,700 years over the fault 250 km north of the triple junction⁵. With times between events ranging from 90 to 560 years, forecasts over the next few decades are impossible, and without such forecasts it is difficult to make a meaningful statement about the likelihood that last month's mainshock was a foreshock to a modern great earthquake on the fault. The last event, 300 years ago, has been geologically observed from northern California to Washington, and so could have had a magnitude of 9. In contrast last month's $M=6.9$ earthquake relieved strain along only about 30 km of the subduction zone and would increase strain only along a smaller portion to the north.

The effect to the south on the closest portion of the San Andreas fault should be insignificant because that segment of the fault is far from recovering the slip that occurred in 1906, hence any small amount of stress added by this sequence would not place the fault close to failure. The distant San Francisco Bay area has faults closer to failure but is too far away to be affected by the events at the Mendocino triple junction.

The mainshock of 25 April produced a surprising peak acceleration of over twice the acceleration of gravity, which lasted 0.1–0.2 seconds as recorded on solid rock just southwest of the epicentre⁶. If no instrumental problems are found, then the unusually large acceleration, which cannot be attributed to amplification from weak near-surface materials, could have a variety of explanations with different implications. Unusual site-specific amplification could be due to the instrument's location on a small knife-edge ridge produced by a road cut into a steep hill, and a fracture



Simplified block model (not to scale) of the three plates north of the Mendocino triple junction. The model is seen through the California coastline and major faults. Shaded areas show the sides of parts of the plates below the surface. The view is from above and to the southeast.

est accelerations ever recorded on a hard-rock site, while producing surprisingly small amounts of ground failure.

Two of the faults that meet at Mendocino are well-known and feared. To the south is the San Andreas fault, expected to fail in the next great earthquake in San Francisco, where the North American plate and Pacific plate are slipping sideways (roughly north-south) past one another. To the north is the Cascadia subduction zone, where part of the Pacific sea floor (the Gorda plate) is diving beneath the northwestern coast of America. Seismically, this fault has remained quiet for at least two centuries, but the fear is that it is storing up strain that might be released in a single catastrophic earthquake¹: the geological record seems to bear out this fear, apparently revealing the occurrence of a

most of the Gorda plate and led Wilson³ to conclude that the Gorda is not a plate but a deformation zone that forms a diffuse southern boundary of the Juan de Fuca plate to the north. The magnitude (M) 6.9 mainshock on 25 April at 18.06 GMT reflects this complexity. This earthquake moved part of the Gorda plate underneath North America on a plane that dips down to the northeast, although most of the Cascadia subduction zone most likely dips to the east. This orientation can be explained by the curvature of the subduction zone as it approaches the triple junction⁴ and agrees with geodetically measured strain before the event (M. Lisowski, personal communication). The $M=6.5$ aftershock 17 hours later was contained within the Gorda plate and was produced by lateral motion across a vertical plane oriented

a few metres from the station and a nearby landslide could indicate that local ground failure contributed to the waveform. Aftershock recording on portable instruments and geological mapping around this site, already underway, should help resolve the question. Or perhaps this record is simply the result of having an instrument directly over a large event, and it will yield new insights into the source process of earthquakes. Fortunately the pulse's short duration reduces its relevance to earthquake engineering of structures, although it could affect industrial facilities. A surprising aspect is that this large earthquake produced little liquefaction and few block landslides.

The $M=6.5$ aftershock produced an unusually large amount of energy in the frequency range 0.3–3 Hz when compared with the other two large events (G. Beroza, personal communication). As buildings respond strongly to these frequencies, this event was felt further away than the mainshock by people indoors and produced additional damage around the epicentre. The cause may be that the aftershock released an especially large amount of stress from a fairly small area on a fault.

Interestingly, instruments in this area have previously made a small but significant contribution to the discipline of seismology. Bosch–Omori seismographs run by a storekeeper in Ferndale (and still operating at the Ferndale Museum) led to his discovery in 1934 that ocean waves are a principal source of noise on seismograms.

Despite the exciting science, my main feeling after a visit to the area around the epicentre was dismay. Most of the damage I saw was sustained by older buildings that could have been easily improved to increase their shear strength. Particularly disturbing was damage to the poorly braced Petrolia Fire Station: this hindered efforts to fight a fire that consumed the tiny village's combination general store, gas station and post office. As our understanding of earthquake hazards increases we must ensure that it reaches the people who need it. □

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Receding from our grasp

Kevin Hurley

SPORADIC flashes of γ -ray radiation in the sky have long puzzled astronomers: they rarely seem to be related to known astronomical objects, and it is hard to know even whether they originate within our Galaxy or whether they are more powerful events further away in the Universe. Data from the satellite-borne Compton Gamma-ray Observatory¹, which should have clarified matters, are in fact confusing matters further. On page 140 of this issue², Fenimore *et al.* opt for the idea that γ -ray bursts originate far beyond the confines of our Galaxy and that puzzling variations in their properties are due to evolution over cosmological time.

If the Compton results¹ are taken at face value, then the resolution of this two-decade-old problem would appear to be receding from our grasp at a rate consistent with the Hubble law of cosmological expansion — a simile which is perhaps appropriate in the light of the discussion in this issue². Although the mystery of γ -ray bursts was far from solved several years ago, some pieces of the puzzle appeared to fall into place and strongly indicated the sources to be magnetized, compact objects in our Galaxy, most probably neutron stars.

But one piece which stubbornly refused to fit was their spatial distribution: it was isotropic, consistent with a spherically symmetric distribution, whereas neutron stars are thought to congregate in the disk of our Galaxy, like the Milky Way itself. It was generally assumed that the sensitive, large-area BATSE (Burst and Transient Source Experiment) detector on the Compton Observatory would record weaker bursts whose spatial distribution would reveal their origin, and that this might well turn out to be in the galactic disk.

One year after launch, it is apparent that this is not to be. Not only is the distribution still isotropic, but there is also evidence that BATSE is sensitive enough to detect bursts to their distance limit (whatever it may be) and beyond. Hence the full volume of bursts is being sampled. While none of the previous evidence in favour of a compact object origin has yet been contradicted, none of the newer data support this idea directly either. Not surprisingly, new ideas on cosmic γ -ray bursts have been proposed, and some, making use of the fact that one of the few truly spherical distributions centred around us is the Universe itself³, involve a cosmological origin.

In the zoo of high-energy astrophysical objects, γ -ray bursts occupy a niche unto themselves. They erupt for perhaps 10

seconds, emitting photons with energies up to 100 MeV, then abruptly disappear at all wavelengths. No quiescent counterpart has ever been identified with certainty, and the distances to bursters are consequently unknown. Statistical studies of the burster population have been carried out for years in an attempt to prove or disprove a galactic origin. The simplest involves examining the spatial distribution to detect a concentration about a point such as the Galactic Centre or a nearby galaxy, or a plane such as the Milky Way. None has been detected. Another is the source count method, enumerating the number N of sources with intensity greater than P . Simple geometrical arguments can be used to show that the line $\log N$ – $\log P$ has a slope of -1 for a disk distribution, whereas with a spherical distribution in euclidean space, the slope is -1.5 .

But because γ -ray bursters are transient sources, their detection is subject to instrumental selection criteria, leading to uncertainty in N and undercounting of the weakest sources; and because the detectors are not bolometers (they do not detect total brightness), there can be considerable uncertainty in P , particularly when counts from different instruments are combined. In addition, source-count statistics must be corrected for instrumental sky coverage, as a survey insensitive to bursts from the galactic disk is obviously unlikely to deduce a $\log N$ – $\log P$ slope of -1 .

To the uncertainties inherent in the method, source-count statistics were consistent with a spherical distribution for the stronger and presumably nearer sources, but this fact did not contradict a galactic origin, as it could be argued that it would be the distant, weaker and undetected sources which would be clustered within the disk. With the newer data from BATSE, all of this has changed. The $\log N$ – $\log P$ curve has now been reliably sampled at source strengths an order of magnitude below previous limits, and it is apparently inconsistent with a downward extrapolation of the old -1.5 slope, as Fenimore and colleagues show in their Fig. 1 (page 140).

Taken with an isotropic spatial distribution, this is difficult to reconcile with the geometry of our Galaxy. It was pointed out very early in the game⁴ that a bend in the $\log N$ – $\log P$ curve might signal cosmological effects. For example, faint, distant sources could be receding rapidly (owing to cosmological expansion), redshifting the γ -rays to longer wavelengths. This would reduce the apparent burst strength P and the

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observed burst frequency, decreasing N . But in fact, say Fenimore *et al.*, the present inconsistency cannot be explained by these effects alone.

To arrive at this conclusion, the authors have had to combine two sets of data. Although BATSE is detecting the numerous weaker bursts, it has not been in operation long enough to detect many of the rarer, strong events to complete the $\log N - \log P$ curve at the high- P end. This gap is filled by the data from a small instrument on Pioneer Venus Orbiter which has been in operation for over a decade. (In fact, because the Pioneer experiment views practically the entire sky, whereas BATSE's view is always obscured by Earth, the two databases may never be equal for strong events, although they may come close.) The two experiments do not cover the same energy range and have very different sensitivities, so combining the two curves is a contentious business. But even with the uncertainties inherent in the method, the two curves apparently

cannot be reconciled.

So where does the answer lie? Before assuming that γ -ray bursters are cosmological, two other possibilities can be considered. The sources could be spherically distributed around the Sun's locality in the Galaxy and still fit the statistical results, but there are few ideas currently about what might generate γ -ray bursts in our cosmic backyard. Stepping back farther, a distribution about the galactic halo would be consistent with the statistics and possibly with the neutron-star hypothesis⁵, but unlike other known halo distributions, the burster distribution would have to have a minimum core radius of 18 kiloparsecs (on the galactic outskirts) for it not to produce an excess of sources towards the Galactic Centre⁶.

But if the bursters are cosmological, one possibility suggested by Piran⁷ is that they are the product of merging neutron stars in binary systems in galaxies at redshifts of around 1, whose radiation has travelled 20 billion light years.

This would preserve the idea that they come from compact objects and would give the requisite N and P providing that cosmological evolution has changed the merging population. The conclusion of Fenimore *et al.* is similar: that whatever the sources are, if they are beyond our Galaxy, then cosmological evolution has decreased their number or strength. It certainly seems, however, that the answer to this quandary, like the bursters themselves, may be further away than we thought. □

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OBITUARY

Isaac Asimov (1920–1992)

ISAAC ASIMOV, one of the great explainers of the age, died on 6 April, aged 72.

Born in Russia, just after the revolution, of Jewish parents (although he speculated that the name might be Islamic, meaning son of Hassim, with Uzbek roots), Asimov emigrated with his family to Brooklyn at the age of 3. His early life revolved around his father's candy store, where he taught himself to read with the magazines on the shelves and first encountered science fiction. He received a PhD in chemistry at Columbia, became professor of biochemistry at Boston University Medical School, and was co-author of the text *Biochemistry and Human Metabolism*. But he became world famous for his work in science fiction and the popularization of science.

Like T. H. Huxley, Asimov was motivated by profoundly democratic impulses to communicate science to the public. "Science is too important", he said paraphrasing Clemenceau, "to be left to the scientists." It will never be known how many practising scientists owe their initial inspiration to a book, article or short story by Asimov — nor how many ordinary citizens are sympathetic to the scientific enterprise for the same reason. M. Minsky, one of the pioneers of artificial intelligence, was first motivated by Asimov's robot stories (conceived to illustrate human/robot partnerships and to counter the prevailing notion, going back to *Frankenstein*, of robots as necessarily malign). At a time when science fiction was mainly

devoted to action and adventure, Asimov introduced puzzle-solving themes that taught science and thinking along the way.

A number of his phrases and ideas have insinuated themselves into the culture of science — his description of the Solar System as "four planets plus debris", for example, and his notion of carrying icebergs from the rings of Saturn to the arid wastelands of Mars.

His output was prodigious, approaching 500 volumes, always in his characteristically straightforward and plainspeaking syntax. The Science Fiction Writers of America voted *Nightfall* the best science fiction short story of "all time". He received prizes from the American Chemical Society and the American Association for the Advancement of Science, and over a dozen honorary degrees. His interests were not restricted to science: his legacy includes two-volume guides to Shakespeare and the Bible, and a thick commentary on Byron's *Don Juan*. The *Foundation* series on the decline of a galactic empire was based on a close reading of Gibbon's *Decline and Fall of the Roman Empire*, a principal theme being the effort to keep science alive as the Dark Ages rolled in.

Asimov spoke out in favour of science and reason and against pseudoscience and superstition. He was a founding fellow of the Committee for Scientific Investigation of Claims of the Paranormal, and President of the American Humanist Association. He was not



afraid to criticize the US government and was deeply committed to stabilizing world population growth.

As someone born in poverty, and possessed by a lifelong passion to write and explain, Asimov by his own standards led a successful and happy life. In one of his last books he wrote that "my life has just about run its course and I don't really expect to live much longer". However, he went on, his love for his wife, the psychiatrist Janet Jepson, and hers for him sustaining him. "It's been a good life, and I am satisfied with it. So please don't worry about me."

I don't. Instead, I worry about the rest of us, with no Isaac Asimov around to inspire the young to learning and to science.

Carl Sagan

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Horse scents

STALLIONS competing for mates judge the form of a rival male not only by listening carefully to its whinnies but by sniffing its faeces as well (D.I. Rubenstein and M.A. Hack *Evol. Ecol.* **6**, 254–260; 1992). Whinnies indicate dominance status, from the horse's mouth, as it were, irrespective of whether the two horses are familiar with each other or not. Faecal smell, though, reveals the identity and home turf of a particular horse, which can then be associated with the outcome of previous contests. Redundant signalling in one species is unusual, but may be a belt-and-braces solution for animals with large and overlapping ranges. The consequent saving on wasteful physical combat echoes Churchill's dictum that "to jaw-jaw is better than to war-war".

Under pressure

HYDROGEN is a singly valent element, as is sodium, so it stands to reason that when transformed into a solid — by pressure — it should become metallic. H. K. Mao and R. J. Hemley claimed three years ago to have achieved this remarkable state of matter. But because the pressures involved are so high, false signals from the apparatus may have confused the issue. Mao, Hemley and M. Hanfland now reply to a charge that the small quantity of powdered ruby (aluminium oxide), included in their cell to calibrate the pressure, becomes reduced by the hydrogen to create traces of aluminium metal. The authors have repeated their experiments with different proportions of ruby, and find that the indicators of metallization (such as increased optical reflectance) are unchanged (*Phys. Rev. B* **45**, 8108–8111; 1992). These are experiments at the extremes of techniques, however, and many remain to be convinced that metallic hydrogen has truly been found.

Tackling TB

TUBERCULOSIS is back with a vengeance in places such as New York City, where the disease is reaching epidemic proportions. But as A. M. R. Chowdhury *et al.* point out in *The Lancet* (**339**, 1181–1182; 1992), in the developing nations it has never been away. In Bangladesh, estimates of the percentage of people testing positive for the TB bacillus have remained at a stubborn 0.5 for over a quarter of a century, in part because TB control has been urban-based — most Bangladeshis live in rural areas and so three-quarters of patients fail to complete their course of treatment. Chowdhury *et al.* describe the results of taking treatment measures to the people who need them, in the form of a community-based programme. Completion rate was an impressive 60 per cent, and the cost per case (\$108) comparatively low. Plans are being laid to expand the programme.

Eukaryotic initiation rites

Joachim J. Li and Bruce M. Alberts

INITIATION of chromosomal DNA replication, which defines the transition from the G1 to the S phase of the cell cycle, is a pivotal event in the life of a eukaryotic cell. It occurs with precise timing and is carefully coordinated with other events in the cell cycle. Moreover, S phase is normally an all-or-nothing event: the initiation process leads to replication of all of the DNA once and only once, followed by division of the cell in M phase. Attempts to understand how these controls are achieved have been limited by our ignorance of the initiation machinery in eukaryotic cells. Partly for this reason, identification of this machinery has become a holy grail which has attracted researchers ever since 1979, when the first eukaryotic replication origins were discovered in the yeast *Saccharomyces cerevisiae*¹.

As reported by Bell and Stillman on page 128 of this issue², the long and arduous search now seems to have paid off with the purification of a large multiprotein complex. The complex binds to the yeast replication origin and appears to wrap the DNA around itself. Also reported in this issue³ is supporting evidence from Diffley and Cocker which suggests that this complex actually binds to the origin *in vivo* (see page 169).

From studies on bacterial⁴ and viral^{5,6} systems it was predicted that initiation of chromosomal DNA replication in eukaryotes would involve an initiator protein that binds to the origin of replication, a *cis*-acting DNA sequence that specifies the site of initiation. For reasons that are still unclear, defining such origin sequences has proven to be a daunting task in most eukaryotes. Only in *S. cerevisiae* have the replication origins been sufficiently characterized to encourage a search for an initiator protein based on its predicted origin-specific DNA binding.

Yeast origins¹ are some 100 base pairs in length and have two essential elements, termed A and B. The A element is primarily composed of an 11 base-pair consensus sequence, the only sequence common to all origins from *S. cerevisiae*. Although normally a required part of every yeast chromosomal origin, the B element can be replaced by several copies of the A element when origin function is tested on plasmids. These properties of the A element recommend it as the most likely binding site for an initiator protein in yeast.

Despite evidence suggesting that a protein actually occupies this site *in vivo*⁷, the search for such a protein has frustrated researchers for many years.

Several groups have recently discovered an activity that binds to one of the single strands of the A region^{8–10}. Its function is not clear, however, especially because purified preparations of this DNA-binding protein exhibit only weak sequence specificity⁸. At any rate, if the protein participates in replication, its action would presumably require the prior conversion of the closed duplex origin into an open single-stranded form. Hence researchers have maintained faith in the existence of an initiator protein that binds to the duplex A element as a first step in the initiation of yeast DNA replication.

This faith has now been affirmed by the work of Bell and Stillman. They have identified and purified a DNA-binding activity that recognizes the A element as a double-stranded sequence. This activity is associated with a tight complex of six proteins called the origin recognition complex (ORC). Identifying this activity was a remarkable feat, in that it could not be detected until several steps into the purification. In addition, ORC could not be seen by gel retardation assays, and its footprint was only observable in the presence of ATP.

Using a panel of A-element point mutants, Bell and Stillman established a tight correlation between the ability of the mutant DNAs to bind ORC *in vitro* and their ability to function as plasmid origins *in vivo*. This correlation is complemented by the experiments reported by Diffley and Cocker in which they detected the signature of the ORC footprint *in vivo*. Together, the two sets of results argue strongly that an initiator protein from eukaryotic cells has finally been captured. That ORC forms a similar footprint on several different yeast origins further suggests that the same initiation machinery can work on many distinct replication origins scattered throughout a eukaryotic genome.

Additional analysis of ORC may soon provide more evidence of its role in initiation. Because of its many subunits, one might expect it either to exhibit several different biochemical activities or to be regulated in a complicated way. It would be encouraging to find that ORC exhibits biochemical activities that are associated with known bacterial⁴ and viral^{5,6} initiation complexes (for example duplex DNA unwinding, helicase activity and ATP hydrolysis). These activities have not been detected to date. Although this might be explained by trivial reasons, it is conceivable that the biochemical activities of a protein involved in such a highly regulated event

are maintained under tight control. Learning how to unleash them *in vitro* could therefore reveal how this protein is regulated *in vivo*. Ultimately, direct demonstration that ORC initiates yeast DNA replication will rely on the establishment of an *in vitro* system in which exogenous templates containing a yeast origin can be replicated. That is the next technical challenge to be tackled, and it should be greatly facilitated by the ability to purify large quantities of ORC.

Assuming that ORC proves to be the initiator protein, at least four ramifications arise. First, a great deal of effort in the cell-cycle field is directed towards understanding the events that lead from the activation of cyclin-dependent kinases in G1 phase to the initiation of DNA replication at the G1/S boundary. Identification of the initiator protein allows us to replace the abstract notion of a G1 to S transition with a defined molecular change, which provides an important tool for working backwards to define and order the activating events in G1. For example, genetic analysis in *S. cerevisiae* has identified a series of cell division cycle (*cdc*) genes whose actions appear to be interposed between the activation of the CDC28 kinase (the *S. cerevisiae* homologue of p34^{cdc2} kinase) and the initiation of DNA replication^{11–14}. How these genes promote DNA replication is unclear. We can now ask how the product of each of these genes affects the different components of the initiator protein complex. Some of the products may be involved in the synthesis, assembly, modification or activation of ORC subunits, whereas others may serve as actual subunits of the complex.

The identification of this putative initiator protein also promises to shed light on a second biological mystery: how initiation of DNA replication at each

origin is limited to only one round per cell cycle (discussed in ref. 15). Explanations of how initiation occurs once and only once must ultimately converge on the actions of the initiator protein. With the protein in hand we can now test some of the models that have been proposed. For example, is the initiator protein inactivated during each initiation event, or is the freshly replicated chromosomal template altered to make it impervious to the continued action of the initiator? Moreover, once the initiation machinery has 'fired', what happens to the initiator protein as the system is reprimed for the next cell cycle? By following the fate of the protein during the replication reaction and throughout the cell cycle, we can expect to learn how the cell prepares itself for a new round of initiation and how it prevents those preparations from occurring prematurely.

A third question concerns the time within S phase at which a eukaryotic origin will initiate DNA replication. This question has been examined most rigorously in *S. cerevisiae*¹⁶. Although many yeast origins initiate at the onset of S phase, a few appear to delay their action until later in S phase. From recent work it seems that proximity to a telomere at

the end of a chromosome is sufficient to induce a yeast origin to 'fire' late. The discovery of ORC should allow us to rephrase the problem in terms of telomeric effects on ORC binding and ORC activation.

Finally, complete characterization of ORC and each of its protein components should provide useful biochemical and immunological tools for identifying similar components of the replication apparatus in higher eukaryotes. Indeed, the discovery of homologues to ORC and analysis of their DNA-binding sites could provide a shortcut to defining replication origins in higher eukaryotes.

Work remains before we can confirm the function of the origin recognition complex, but its discovery has moved research in eukaryotic DNA replication into a promising new phase. The field is now poised to emerge from the shadows of its more developed bacterial and viral counterparts, providing us with the opportunity to solve salient problems specific to eukaryotic cells. □

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Fashioned by fire



The series of fires that swept through Yellowstone National Park in the summer of 1988 exposed the mountain slopes to the destructive effects of the intense local thunderstorms. On page 147 of this issue, G. A. Meyer *et al.* show that erosion after such fires follows a pattern that has sculpted the landform of Yellowstone over the millennia. The authors looked at the stratigraphy of alluvial fans in two creeks, and identified and dated 18 fire-related debris-flow runouts and another 17 fire-related sediment runouts from the past 3,500 years. Extended periods of drought (350–100 BC and AD 1000–1200) feature strongly in the stratigraphic record. But, as the recent experience shows, a single dry summer can leave its mark, so that rapid climate variability may equally be implicated. The picture (from August 1989), of a region above Soda Butte Creek, shows another characteristic of the fire damage — the speed with which vegetation can recolonize and restabilize the soils.

R.P.

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Not only gone with the wind

Peter D. Moore

THE value of detailed study of apparently well-defined ecological processes is evident in new work reported in *Ecology*¹ by S. B. Vander Wall. He shows that even among seeds (such as those of pine) regarded as wind-dispersed on the basis of their morphological structure, secondary dispersal by animals can be crucial to effective seed dissemination, and to ultimate germination and establishment.

Population mobility is a valuable asset to a species, especially under conditions of rapid environmental change when the suitability of a given habitat may alter. Among mobile species, the problems to be faced are mainly those of overcoming physical barriers, but for most plants population movement depends also on the efficiency of fruit or seed dispersal. In studying this aspect of plant population biology, many ecologists have been content with relatively simple analyses of dispersal mechanisms, often classifying them into a series of types (wind, water, animal vectors and so on). But careful examination of individual cases, such as that described by Vander Wall, demonstrates that the real world is more complicated than that.

The involvement of animals in the secondary dispersal of seeds is by no means a novel observation. In violets (*Viola* species), for example, animals are often implicated in this second stage². Initial dispersal is usually effected by a violent explosion of the fruit as the drying capsule splits into three elastic valves and ejects the ripe seeds.

Scatter

Although this results in a scatter of seeds only tens of centimetres from the parent plant, it does at least take the new generation out of the immediate influence of the old. The seeds of many species, however, are invested with a caruncle containing oily material that is much prized by ants, which gather the seeds and transport them to their nests. After consuming the caruncle (which does not damage the seed embryos), the remainder of the seed is discarded and may subsequently germinate. A two-stage dispersal of this kind raises the question of whether the plant may be better served by missing out the first (relatively inefficient) stage and so give the ants a better chance of finding the entire, seed-packed capsule, rather than having to forage for scattered individual

seeds. On the other hand, the initial explosive dispersal can provide a useful backup in the event of the failure of the ant-mediated stage.

A similar double strategy dispersal has been described by Janzen³ for guanacaste tree seeds (*Enterolobium cyclocarpum*) in Costa Rica. Here the seeds are consumed initially by horses and pass unharmed through the gut to be deposited in the faeces. The collections of seeds in dung piles are then the focus of the foraging activity of a seed-eating



The yellow pine chipmunk, an agent of seed dispersal.

rodent, the spiny pocket mouse (*Liomys slavi*), which caches seeds in its tunnels, and Janzen has been able to show experimentally that seeds are more effectively harvested by the mouse from scattered small (2-litre) dung piles than from large (8-litre) ones. This dispersal process has the advantage over that of *Viola*, in that even the first stage is efficient in terms of distance travelled, but the seeds are still deposited in groups and are thus more easily harvested by foraging rodents. The relative value of what Howe⁴ has termed "clump" and "scatter" syndromes in the dispersal of seeds is evidently related to whether or not a secondary vector is involved in the final disposition of the propagules.

Following the movements of individual seeds during dispersal, especially secondary dispersal by animal vectors, presents many practical problems, and Vander Wall¹ has used radioactive isotopes in his studies of the Jeffrey pine (*Pinus jeffreyi*) in Nevada. Using seeds soaked in scandium-46 (a γ -emitter with a half-life of 84.5 days), he laid out groups of eight seeds at different distances from a pine tree, simulating densities expected from wind dispersal and extending out to 12 m from the tree. Each seed was marked with its source

location and the experiments were run for between two and five days, after which the seeds were relocated using a γ -radiation detector. Yellow pine chipmunks, together with other small mammals, had been observed gathering pine seeds in the area, and it turned out that over 95 per cent of the experimental seeds disappeared over the test period. With the aid of the detector, 128 caches of seeds were found in chipmunk tunnels, accounting for 54 per cent of the lost seeds.

Distance

Whereas the wind-dispersal model predicted that most seeds would fall within 12 m of the tree, some caches, the contents of which ranged from 1–13 seeds, were found at distances of over 60 m. The wind model does not allow for high wind speeds, which could permit wider dispersal, but it was realistic in its prediction that seeds would be concentrated close to the source tree. The secondary dispersal in this case is effective in extending the mean dispersal distance of seeds, thereby increasing the likelihood that seeds would be deposited in suitable locations away from the immediate shade of their parent.

A further beneficial effect of the secondary dispersal is the burial of the seeds. In an experiment designed to determine the comparative effects of burial and superficial deposition, rodents were excluded from certain areas and some seeds were left on the surface while others were buried in a manner intended to simulate the activity of chipmunks. The following spring, the only surface-deposited seed to germinate was one that had subsequently been buried by ants. All the remainder failed to germinate, whereas 55 per cent of the buried seeds produced a seedling. It seems that the pine gains not only from the additional dispersal provided by a secondary rodent vector, but also by the treatment received at the paws of this mammalian agent. Obviously, many of the seeds in such caches will be consumed by the chipmunks, but occasional chipmunk death or forgetfulness means that a significant proportion survive to germinate another day. □

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Slimline magma chambers

Sherman Bloomer and Peter Meyer

THE 60,000-km-long oceanic rift system marks the site at which nearly two-thirds of the Earth's crust is produced. The volcanic carapace of that crust, and its plutonic underpinnings, are thought to develop from shallow magma reservoirs or 'magma chambers'. The shape, size and physical behaviour of these chambers determine much of the geochemical, geophysical and hydrothermal character of oceanic crust. For most of the past 25 years, oceanic magma chambers have been viewed as large, predominantly liquid reservoirs of various sizes and shapes.

Sinton and Detrick, in the *Journal of Geophysical Research*¹, now propose a very different view. Combining field observations with geophysical, geochemical and computational data, they develop a model of magma chambers consisting principally of narrow, hot crystal-melt mush zones overlain by very thin lenses of pure melt.

The existence of some type of crustal magma chamber has been assumed since the first observations of oceanic lavas. The petrographic and chemical characteristics of most of these lavas show that they have been modified by crystallization or mixing in a crustal environment. Studies of the thick gabbroic sections associated with ophiolites (rare blocks of ocean crust uplifted onto land) were taken to indicate the existence of large, steady-state, melt-dominated magma chambers whose size and shape varied with spreading rate and thermal regime^{2,3}. The generation of the crust was likened to crystallization around an 'infinite onion' of silicate melt².

Contradiction

A growing number of petrological and geophysical observations, however, are at odds with this intuitively satisfying model of magma chambers. The fine-scale geochemical segmentation of fast-spreading ridges⁴, the complexity of trace element systematics in many ocean ridge lava suites⁵, and the evidence of extensive magma mixing at slow-spreading ridges⁶ are all difficult to reconcile with ideas of large, well-mixed reservoirs beneath the ridges. Sinton and Detrick suggest instead that mid-ocean ridge magma chambers are composite structures dominated by a hot transition zone consisting of a semi-rigid to rigid crystal framework with only small amounts of interstitial melt (less than 40 per cent at its inner boundary, the rigidus, and only 1–2 per cent at its outer boundary, the solidus). These transition zones range from the solid wall rock to a

core of crystal mush, in which the proportion of melt is sufficiently high for the magma to move as a highly viscous fluid (see figure).

Pure-melt segregations develop only in faster spreading ridges (spreading at more than 50 mm yr⁻¹) where magma supply rates are high enough to produce a thin melt lens above the crystal mush. This melt lens is continuous along the ridge axis, but has a very high aspect ratio, with lengths of the order of tens of kilometres but thicknesses of only tens or hundreds of metres. Slow-spreading ridges do not have sufficient magma supplies to segregate a melt lens, and their magma chambers consist only of a narrow (2–4 km) transition zone with a transitory crystal mush core.

This model has its roots in a number of earlier studies of magmatic systems, which have increasingly emphasized the nature of magma as a mixture of melt and crystals. For example, Natland⁷ had suggested that the diverse lava compositions erupted at the East Pacific Rise require a composite magma chamber, in which a large melt reservoir acts as a buffer for various batches of magma, and isolated pockets of melt (most likely in the crystal-rich margins of the chambers) produce highly fractionated ferrobasalts.

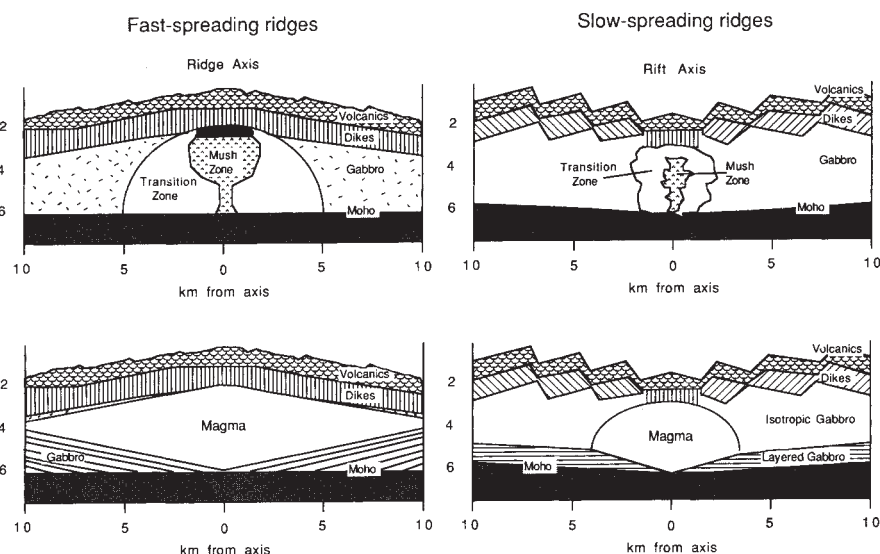
Field and computational studies of magmatic systems at Kilauea, Hawaii, and in island arcs emphasized the importance of crystal-melt mixtures in

magma reservoirs: Helz *et al.*⁸ have documented the diapiric (buoyant) extraction of melt from crystal mushes in the Kilauea Iki lava lake; and the concentration of late-stage liquids from a rigid crystal-melt zone by deformation has been documented in gabbroic sections recovered from Ocean Drilling Project site 735B on the Southwest Indian ridge⁹. Marsh¹⁰ has pointed out that magma chambers, as they must range from liquidus to solidus conditions, consist of three parts — a rigid outer crust with greater than 55 per cent crystals, a mush zone with 25–55 per cent crystals, and a melt zone with less than 25 per cent crystals.

Characterization

The dimensions and character of oceanic magma chambers have been tied down by a number of geophysical studies, most in the past five years. Rosendahl¹¹ was perhaps the first to maintain that the magma reservoir appears to be a wedge-shaped zone below the axis, containing no more than 30 per cent melt. Recent seismic reflection studies have delineated a narrow (1–4 km), bright reflector 1–2 km beneath some intermediate and fast-spreading ridge segments¹². The character of the reflections from this boundary, and the lack of clear basal reflections, suggest that this reflector marks a melt-dominated lens that is only a few tens or hundreds of metres thick¹³; it is, however, laterally continuous for tens of kilometres along the ridge, even across ridge segments with marked geochemical discontinuities.

This melt lens lies above a larger low-velocity zone that extends from the



Comparison of the crystal-rich magma chambers of Sinton and Detrick¹ (top) with melt-dominated magma chamber models like those of Cann² and Pallister and Hopson³ (below). Note that 'magma' in the latter case is taken, at least implicitly, to be melt-rich. The pure melt lens in the fast-spreading case is shown in black. The mush zone has over 25 per cent crystals and behaves as a highly viscous fluid; the transition zone has more than 60 per cent crystals and behaves as a rigid or semi-rigid material. Note the overall smaller size of Sinton and Detrick's magma chambers and the very small proportion of pure melt in them.

melt lenses to the base of the crust, where it is 10–12 km wide¹⁴. No similar axial structure has been seen at slow-spreading ridges. In fact, seismic studies indicate that at least some portions of the crust are brittle to depths of 8 km, precluding the presence of substantial magma chambers¹⁵.

Sinton and Detrick assemble diverse observations in constructing the model, the first comprehensive new hypothesis for oceanic magma chambers in many years. Their model differs from previous ones in the small size of the chambers, the very large proportions of the chambers which are occupied by rigid or highly viscous crystal-rich assemblages, and by the authors' effort to relate a number of petrological and geochemical anomalies to the structure of the magma reservoir.

Magmatic processes in their model are controlled by two types of mixing and two types of fractionation. Fractionation can occur within the crystal-dominated mush zone or transition zone (a case approaching the *in situ* crystallization of Langmuir⁵) or within the melt lens (a case approaching open- or closed-system Rayleigh fractionation). Mixing can occur within the melt lens, which functions as a buffer homogenizing melt stored in the lens with inputs of melt from the mantle or the crystal mush zone. Mixing can also occur in the crystal mush zone. Liquids from the transition zone may be remobilized by deformation and may also be involved in mixing in the mush zone or the melt lens.

Magmatic processes at intermediate and fast-spreading ridges are dominated by mixing and fractionation within the melt lens. The lens homogenizes individual batches of melt from whatever source and may serve as a filter to remove xenocrysts from deeper in the mantle. The aspect ratio of the pure-melt horizon is such that mixing within the lens is efficient perpendicular to the ridge but is severely restricted parallel to the ridge axis, which explains geochemical segmentation which is unrelated to tectonic segmentation.

Slow-spreading ridges lack a melt lens, and the chemistry of their eruptives is controlled by mixing and fractionation within the crystal mush zone. Only when periodic influxes of magma from the mantle bring enough melt into the system do eruptions occur. Consequently, volcanics at slow-spreading ridges are distinctly less fractionated than those from faster ridges, and carry the mark of mixing of crystals and interstitial melt from the mush zone. Extreme fractionation at slow-spreading ridges occurs only in the transition zones; the rigidity of this zone precludes eruption of these fractionated compositions and their

migration and emplacement is strongly controlled by deformation at the ridge axis.

Sinton and Detrick's model provides an intriguing point of departure for future work. There are two particular questions raised. First, it is not at all clear how this kind of magma chamber can lead to the development of layered gabbros. Phase and cryptic layering are prominent features of ophiolite gabbroic sections and the available evidence indicates that similar layering exists to a degree in at least some oceanic gabbro suites. Some mechanism to account for this layering in these crystal-dominated chambers must be found.

Secondly, it is likely that there is a temporal cyclicity to the chambers drawn by Sinton and Detrick. For example, slow-spreading ridges may oscillate between a state without even a crystal mush zone, to a more inflated state in which tiny melt lenses may develop. Geophysical definition of ridge axes at different stages of magmatic activity should be an important goal, as should a better characterization of the physical properties of the three parts of Sinton and Detrick's magma chambers.

This picture of magma chambers also suggests that a number of new experimental approaches may be appropriate to examine the effects of low-pressure equilibration between primitive mantle melts and the crustal-level crystal mush piles which they invade. These new magma chamber models should provide an incentive for workers in several disciplines to reexamine some of their observations and conclusions about how ocean crust forms. □

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Flying dust

VOLCANIC avalanches can be horrifically devastating. They consist essentially of hot dust fluidized in turbulent air, and can travel at high speeds for many kilometres down slopes of less than 1°. Some pyroclastic flows (in which the dust is fiercely hot) have even been known to travel uphill. Daedalus argues that they cannot merely be passive flows: they must be self-propelled.

A mass of fluidized dust travels with a rolling, shearing motion. The layer next to the ground travels more slowly than those above it: the fastest-flowing topmost layer must continuously spill onto the ground in front of the avalanche. It will entrain the air in its path, forming a bottom layer over which the advancing mass will roll. The captured air will be heated rapidly by the hot dust. Cold near the front, but steadily warming and expanding while the avalanche rolls over it, it will tend to tip the avalanche forward, thus accelerating it. So, says Daedalus, a fluidized mass of hot dust is a sort of mobile Stirling engine, driven along by its own heat.

This bold theory neatly explains the astonishing mobility of volcanic avalanches and pyroclastic dust-flows. It also suggests a new form of bulk transport for powders and granular materials. Daedalus is launching experimental hot-dust avalanches into a long horizontal chute fitted with dips, humps and curves. He is studying their motion, their maximum and minimum stable speed as a function of temperature, their hill-climbing ability and their thermal efficiency as self-propelled vehicles.

Cement manufacturers, fly-ash disposers and so on will welcome the new technology. They will be able to launch a charge of hot powder into a properly designed chute, and it will speed around the works or out to a nearby customer under its own power. But even the hottest powder will cool down and tumble to a halt in a few kilometres. A fuel such as coal dust could be added to the powder; its slowly-released heat of combustion might keep it at avalanche speed for hundreds of kilometres. More elegantly, Daedalus's 'electric avalanche chute' has a large voltage across its two sides. The racing avalanche shorts out the two sides, or causes local dielectric breakdown between them, thus keeping itself hot and maintaining its velocity. Hot bulk powders such as iron ore, soda, cement, sand and gravel could all be avalanched rapidly around the country on cheap dedicated electric chutes. At more moderate temperatures grain, flour, sugar and even cornflakes might acquire a lower but still useful turn of speed.

David Jones

Mount Unzen rumbles on

SIR — Last June's explosive eruption of Mount Unzen stirred up considerable controversy about the ability of Japanese authorities to forecast its behaviour. Although out of the headlines for the past 10 months, Unzen has continued to extrude a large lava dome that looms above the city of Shimabara, shedding rockfalls, pyroclastic flows and mudflows, with no signs of abating. This activity has been monitored more closely than any dome-producing eruption except that of Mount St Helens in the 1980s. A comparison of the two eruptions suggests that the dangers at Unzen are likely to continue for several years.

Although superficially similar, the two eruptions exhibited markedly different styles. The main St Helens dome appeared in October 1980 after most of the explosive activity had ceased, growing slowly and intermittently during October 1986. Throughout Unzen's first year, pyroclastic flows have formed repeatedly by collapse of the dome's oversteepened front, with the dome itself enlarging continuously and vigorously, growing more in 10 months than the St Helens dome did in 6 years. As the St Helens dome grew taller its weight caused an increase in the repose intervals between eruptive pulses, reflecting a delicate balance between the magmatic system and local conditions. Unzen has yet to show any such sensitivity. Finally, the St Helens dome exhibited two lava textures, smooth and scoriaceous, caused in part by variations in dissolved water content. Unzen's lava has been uniformly nonvesicular, indicating a more homogeneous magma source.

A hallmark of the St Helens monitoring programme was repeated collection of high-resolution digital topographic data, allowing more accurate calculations of dome volume than previously possible^{1,2}. Plots of dome growth rate versus time revealed a steady power-law decrease superimposed on episodic emergence of lava. The long-term decline suggested depletion of a relatively small magma source and cyclic behaviour was consistent with a regenerative eruptive process such as crystallization-induced volatile enrichment³. Volumetric measurements at Unzen show steady, rapid production of texturally uniform lava⁴, implying a considerably larger source capable of sustaining a much longer eruption. For comparison, active domes in Guatemala and Kamchatka have grown at rates similar to Unzen's since 1922 and 1956, respectively.

At present, the magnitude of Unzen's pyroclastic activity seems to depend more on the volume of rock-falls than on chemical variations in the magma⁴.

Based on recent inspection of the oversteepened dome face, rock-falls two to three times larger than those yet seen are possible. It would thus be prudent for civil authorities to maintain the current evacuation zones as long as the present extrusion rate continues. Well before Unzen shuts down and life in Shimabara can return to normal, this rate should drop off, accompanied by intermittent effusion of lava with more variable water content, vesicularity and explosivity. Early detection of such trends will require continued funding for a monitoring programme that includes repeated collection of topographic data and photographic documentation.

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Balloon response explanation?

SIR — The remarkable photograph (S. V. Boletzky *et al. Nature* **356**, 199; 1992) of a cirrate octopod hovering at a water depth of 2.88 metres off Lifou Island in the southwestern Pacific raises the question of why the animal prefers transition into a pumpkin shape as a defence mechanism.

The answer could lie in the large 'acoustic size' gained by transition into a spherical shape compared to the normal 'flat' (low acoustic reflective) shape. Marine predators often use high-frequency sound pulses for ranging and location of prey. The sudden appearance of a very large acoustically reflecting object in mid-water nearby would undoubtedly confuse and frighten the predator. I therefore suggest that the octopod 'ballooning' is an effective underwater defence mechanism.

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Diamonds at the K/T boundary

SIR — In our recent report¹ of our discovery of nanometre-sized diamonds at the Cretaceous/Tertiary (K/T) boundary, we suggested that the diamonds may have come from the meteorite that is widely thought to have struck the Earth at that time. We pointed out¹, however, that we could not exclude the possibility that the diamonds were formed by shock metamorphism of carbonaceous target rocks. We have now measured the carbon-isotope ratio of the diamonds and can confirm that they are of extraterrestrial origin.

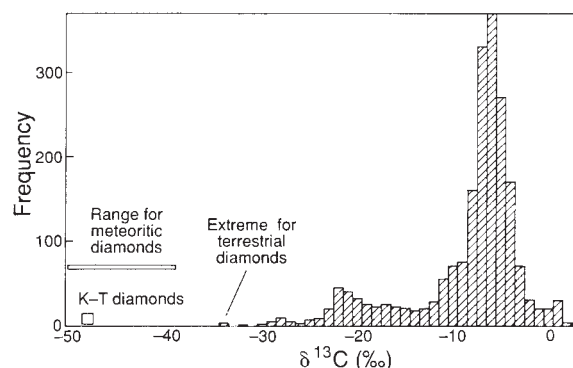
Galimov² summarized the known carbon-isotope ratios for terrestrial diamonds. Although diamonds from different sources differ in $\delta^{13}\text{C}$, the mean value is roughly -7‰ , with the extreme

value -34‰ . Nanometre-sized diamonds from C2 carbonaceous chondritic meteorites have $\delta^{13}\text{C}$ values between -38‰ and -50‰ (ref. 3). Mass spectrometry of a freshly prepared sample of diamonds from the K/T boundary layer of Alberta gave a $\delta^{13}\text{C}$ value of -48‰ . This is incompatible with a terrestrial origin, or with shock formation from carbonate on the impact of a bolide. Such a value is, however, compatible with that found in C2 carbonaceous chondritic meteorites³ and, moreover, seems closer to the general $\text{C}^{13}/\text{C}^{12}$ ratio of the interstellar medium than to the terrestrial norm of 1:89 (ref. 4).

The notion that these diamonds are of extraterrestrial origin is reinforced by the discovery of chromite, spinel and

silicon carbide grains in the K/T boundary, although these too are absent from samples taken 2 cm above or below the boundary; all these species are abundant in C2 carbonaceous chondritic meteorites⁵.

It is perhaps worth noting that the presence of free diamonds and of silicon carbide may indicate that these meteorites, and the bolide whose impact is responsible for the K/T event, were formed under conditions in which the O/C ratio was less than 1,



Frequency histogram of $\delta^{13}\text{C}$ (‰) in terrestrial diamonds (from ref. 2) with the range of values reported in meteorites and the value found in diamonds from the K/T boundary.

whereas the abundant silicate of the refractory planets and of stony meteorites indicates formation under conditions in which this ratio was greater than one⁶. Accordingly, the bolide responsible for the K/T event is unlikely to have been an asteroid, but seems more likely to have been a comet from the remote outer Solar System.

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Peptides bound to MHC

SIR — A. Y. Rudensky *et al.*¹ describe sequence determinations of sets of naturally processed peptides eluted from immunoaffinity-purified murine MHC class II molecules. The authors claim that only about six different peptides would represent most MHC-bound material. Crucial to this claim are quantitative data on the yield of the various peptides, relative to each other as well as relative to the amount of MHC-protein used as a source. Unfortunately, no such data are given¹, challenging readers to make their own estimation.

The 400–500 µg purified MHC-protein used as a source (see Fig. 1 legend of ref. 1) correspond to about 7 nmol. Consequently, one would expect a similar amount of about 7 nmol peptide to be released from this MHC preparation, which corresponds to about 10 µg peptide, assuming an average relative molecular mass of about 1,500 (Table 1 of ref. 1). If, as Rudensky *et al.* suggest, only six peptides together represent 50–70% of all material, any one dominant peptide should yield approximately 1 µg. In our experience, such an amount of peptide would yield peaks on an HPLC trace taken at 210 nm that are at least 20 times higher than those illustrated in their paper (Fig. 1b). Even peptide losses during Centricon filtration of up to 80% would still not explain the extremely poor yield of peptides as suggested by the minute peaks that were apparently found.

Because of the lack of data on the yield of the peptides involved, this consideration seems to suggest that the analysis described by Rudensky *et al.* actually deals with a tiny fraction, perhaps only the most loosely bound, of

the peptides that were originally bound to the MHC preparation. Any far-reaching conclusions based on only a few per cent of the MHC-bound peptides seems inappropriate.

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JANEWAY *ET AL.* REPLY — van Noort correctly points out that yields of peptides eluted from MHC class II molecules reported in our paper¹ are not quantitative. We believe that some weakly bound peptides are lost in early phases of MHC class II purification and concentration, and that a significant amount of material may be lost in acetonitrile precipitation, whereas some of the strongly bound peptide may be lost, as van Noort suggests, during Centricon filtration. We have now documented that individual HPLC peaks contain, in addition to the dominant peptide, small amounts of other peptides. Using electrospray ionization tandem mass spectroscopy, D. Hunt and colleagues (personal communication) now find many peptides of different molecular mass at very low concentrations in a single HPLC peak. Thus, our techniques may under-represent the complexity of peptides associated with MHC class II molecules.

Nevertheless, we do not believe that these considerations undermine the validity of the argument about the complexity of self peptides made in the manuscript² accompanying ref. 1, which van Noort ignores. In this paper, we used the Y-Ae antibody³ to identify a subset of MHC class II molecules that bind a single peptide derived from the I-E α-chain. This antibody binds 12% of MHC class II molecules on B cells, macrophages and dendritic cells³ from strain B10.A(5R) and an equivalent number on B cells from B6 mice (I-E α and Y-Ae negative) transgenic for the I-E α-gene. Recent studies (A.Y.R. *et al.*, manuscript submitted) show that this peptide can occur in different length forms, eluting in distinct HPLC peaks, but that T cells cannot distinguish this variability. Thus, one-eighth of I-A^b molecules in this strain are occupied by one peptide. This peptide binds strongly to I-A^b molecules as well as to I-A^d molecules (A. Sette, personal communication). The yield of this peptide from LB27.4 cells was proportional to its surface abundance^{1,2}.

Finally, the crucial argument in our paper is based on peptide:MHC density requirements determined by other authors^{4,5}. Our data simply limit the complexity of self imposed by these considerations even further. We have never claimed, as van Noort states, that only six peptides are bound to MHC

class II molecules; indeed, we have data that disprove this notion (A.Y.R., manuscript submitted). Thus, whether or not our peptide yields are quantitative, we believe that immunological self is of limited complexity, and that this has important implications for self tolerance. Similar arguments have been made for MHC class I molecules (D. Wiley, personal communication).

It is important that van Noort's correct identification of the nonquantitative nature of our peptide elution strategy is not confounded with his subsequent surmises, which ignore essential data obtained independently of this technique.

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Crystal faces

SIR — J. A. D. Dickson¹ claims to be the first to report crystallographically controlled isotope fractionation of stoichiometric ions in his study of natural calcite, where he found a fractionation of the carbon and oxygen isotopes under the crystallographically differing faces of crystals. But our study² on natural amethyst showed that the ¹⁸O/¹⁶O ratio increases in the order *z*-sector, *r*-sector, *m*-sector of quartz. In a subsequent paper³ on natural smoky quartz, we confirmed the order *r*-sector, *m*-sector.

The sampling methods used by Dickson and by us on crystals of 3–7-cm size are different. Dickson scraped material from the external surfaces of the crystals, which he admits causes sampling errors due to isotope mantle zoning, whereas we took the samples from polished slices of the crystals, where the boundaries between the differently coloured sectors are visible. This enabled us to compare samples from the same range of mantle zones. For millimetre-sized crystals, Dickson took samples from slices with mantle and sector zoning, as we did for our larger crystals, but he could not identify the faces belonging to these sectors.

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Not just desserts

Roy Porter

Rhubarb: The Wondrous Drug. By Clifford M. Foust *Princeton University Press*: 1992. Pp.371. \$35, £27.50.

RHUBARB pie, tart and crumble, even rhubarb wine: the rhubarb that we loathed as children and later came to like as adults is, in the literal sense, a common-or-garden plant. We might naturally associate it with the laxative plant that was championed by Culpeper and other herbalists and that, as the diaries and letters of yesteryear show, was highly popular to relieve constipation. But that, as Clifford Foust's unusual and eye-opening book demonstrates, is only half true and but the tip of the tale.

For one thing, the medicinal and kitchen uses of rhubarb are essentially separate, and its culinary vogue came quite late. Rhubarb was barely known as a dessert before the end of the eighteenth century: old varieties were too bitter. It took new hybrids, improved horticulture (especially the perfection of forcing techniques) and the mass availability of cheap sugar to turn rhubarb into what Foust calls a "culinary craze". (One suspects it became a favourite of the Victorian housewife partly thanks to her punitive 'eat it, it's good for you' mentality.) The West Riding became the great home of the British rhubarb growers, for, like Yorkshirefolk, the plant thrives in damp, overcast conditions, tolerates atmospheric pollution and dislikes strong sunlight.

But the stewed rhubarb that bubbles under pie crusts is not the rhubarb that loomed so large in the traditional doctors' armamentarium. For medicinal purposes, it is not the stalk but the root, firm yet spongy, that is used. And apothecaries' rhubarb (*Rheum officinale*) was derived from several different varieties, with the finest always imported. Ever since the Greeks, rhubarb had been justly famed for its laxative properties, eliminating noxious humours and cleansing the system. Acknowledged as safer than such drastic, scouring cathartics as colocynth and mercury, rhubarb was also preferred for its absence of griping after-effects. And some claimed for it "miracle qualities" too: an eighteenth-century puff declared that "Quintessence of Rhubarb" clears the "skin of Leprosy, Morpew, Scurg, Freckles, Spots, &c. and cures all continual Fevers of what kind soever". Many of the top quack nostrums, such as Morison's Pills and Gregory's Powder,

had a rhubarb base.

The Greeks got their rhubarb from Asia, and thereafter the East remained the chief source. Like opium and coffee, it grew, wild and cultivated, in the Ottoman Empire, and was traded by Levant merchants from ports such as Smyrna. Known by leading botanists such as James Sherard as "rapontic rhubarb", Turkish rhubarb gradually came to be grown in Europe. Dried, powdered and made into pills, syrups and electuaries, this *R. rhaponticum* certainly worked. But there was a widespread suspicion that it wasn't True Rhubarb. This — like

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Rhubarb's culinary vogue came quite late.

all wonders — was believed to stem from the mysterious Orient, somewhere in Tartary or the forbidden Middle Kingdom, glimpsed by Marco Polo and Jesuit missionaries, but as late as 1800 still out of bounds to Western traders.

Some Asian rhubarb deriving from the regions between Siberia and Mongolia filtered to the West, overland by caravan via Moscow and St Petersburg, under a state monopoly set up by Peter the Great. By the 1730s, London druggists were importing about 9,500 pounds of the Russian root annually. In an amusing anticipation of recent events, Catherine the Great chose in 1781 to revoke the monopoly in the name of efficiency, proclaiming free trade in rhubarb.

With Russian rhubarb, the problem was not quantity but quality: frequently it arrived decomposing and worthless. This gave the East India Company the

chance to grab the pick of the trade, shipping the root from India and Chinese trading posts. Rhubarb was responsible for a fair portion of the company's profits — not surprisingly, perhaps, as by 1740 a staggering 16,000 pounds were being imported annually, some for re-export (just how constipated were the British?).

Even so, who knew whether even East India Company rhubarb was the real thing, *R. officinale* or *R. sinicum*? Indeed, did True Rhubarb really exist? Or was it, like the Holy Grail, much bruited but just a figment of the imagination? This enigma explains why so many explorers and diplomats strove to smuggle back seeds, rather than merely ship back the root — although the energetic efforts of the Chinese to keep their 'gold mine' all to themselves long proved successful. As Foust shows, it was not until around 1850 that, botanically speaking, China was opened up by explorers such as Joseph Hooker, pressing up from India through Nepal.

Back home in the United Kingdom, exertions were directed towards perfecting the supply. From the 1750s, the Society of Arts offered premiums for tip-top home-grown medicinal rhubarb; but although, thanks to enterprising market gardeners, domestic cultivation spread, the Urals-derived *R. undulatum* largely grown in Britain never matched expectations. After 1800, efforts concentrated on laboratory analysis of the root, both to clarify its active pharmacological ingredients and to determine the chemical differences between the types. In both areas, hopes exceeded success, as was also the fate of attempts to extirpate adulteration. Some dealers proved remarkably impenitent about the practice of blending with sawdust. Surely, they argued, adulteration was acceptable for exports to foreigners? Was not passing-off English-grown palmated rhubarb (*R. palmatum*) as Asiatic rhubarb — better and more expensive — an act of patriotism? And was not *caveat emptor* the rule?

As will be evident, *Rhubarb* is an appealing book because it skilfully interweaves the threads of science, trade, exploration and medicine. The sheer scale of the operation proves the error of supposing that drugs have become big business only in the present century. Who would have thought that a humble laxative was long one of the pillars of East-West trade? □

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Tools of the astrophysicist

Philip A. Hughes

The Physics of Astrophysics. Volume 1: Radiation. Volume 2: Gas Dynamics. By Frank H. Shu. *University Science Books/W. H. Freeman*: 1991. Pp. 429 (vol. 1)/476 (vol. 2). \$46.50, £29.95 each.

MOST astrophysicists derive immense pleasure from the sense that they are engaged in unlocking a great puzzle, provided with clues often no more substantial than stray photons from a distant source, a knowledge of physics and an ability to describe the Universe through the language of mathematics. Frank Shu, whose interests span the scales from planets to galaxies and who is the author of a noted introductory text for undergraduates, has now written a uniquely detailed yet comprehensive graduate text in which he aims to teach the physics and mathematics that form the essential tools of the astrophysicist.

In volume 1 on radiation, Shu provides a broad introduction to radiative transfer and statistical mechanics and to both classical and quantum theory of radiation processes. In volume 2 on gas dynamics, he introduces kinetic theory and hydrodynamics, devoting about half the volume to laminar and turbulent flow, waves and shocks. The last third covers magnetohydrodynamics and plasma physics.

Those with a 'liberal arts' background may find themselves in need of some additional preparation to use this text, and parts might challenge those whose undergraduate education is limited to the physical sciences. Further, I suspect that there are rather few educators with sufficient depth and breadth of knowledge to feel comfortable teaching the complete one-year course for which the volumes are intended. But many teachers and researchers will want the two volumes on their shelf as a reference source and as a template for courses less ambitious than Shu's.

Despite the high level at which the volumes are pitched, they are clear and practically oriented. For example, in the coverage of the set of equations derived by taking moments of the radiation transfer equation, the justification for closure is presented in a plain, down-to-earth manner, and within the discussion that follows, the use of dimensional analysis for the solar interior gives the reader a true appreciation of the validity of the radiation conduction approximation. The topic of spiral density waves is used to introduce the concepts of wave packet and group velocity, and the



Downtown fishing. The Columbia River flood of 1894 was the largest recorded flood on the river in Oregon history. With a peak flow exceeding one million cubic feet per second, the flood provided angling opportunities in Portland for the boys of the Crystal Palace saloon. This picture is taken from *National Water Summary 1988–1989: Hydrologic Events and Floods and Droughts*, the sixth in a series of reports on the water resources of the United States. Published by the US Geological Survey, price \$39.

'method of characteristics'. This in turn leads to hydrodynamic flows, the classification of partial differential equations and a useful introduction to methods for their numerical solution.

Indeed, one of the most striking characteristics of the book is a linking of ideas and a remarkable absence of compartmentalization: classical radiation theory takes one naturally to plasma effects, and thereby to a chapter on basic plasma physics, while quantum radiation theory leads eventually to a discussion of the nature of chemical bonds. Further down this hierarchy one finds a myriad of edifying digressions: from plasma physics we meet the Cerenkov and Razin effects; from chemical bonds follows a discourse on the nature of the strong force.

There are fluctuations in the depth of treatment. For example, two chapters covering synchrotron radiation guide the student from the basic concepts to an effective working knowledge of the subject; but although the exposition of cosmic dynamo theory is very clear, I think that the student would still have serious difficulty in understanding a modern paper on the subject.

As befits a general astrophysics text, it is not encumbered with extensive references. Two references usually follow each chapter heading to guide the reader to original and more detailed discussions. Citations of textbooks are gathered together in a bibliography, but

journal articles are not, making it slightly difficult to find half-remembered references. And given that the text will surely become a standard, it is a pity that it continues to perpetrate the usage of cgs units, just when the astronomical community is beginning to adopt SI units.

Both volumes contain nontrivial sets of problems that introduce additional material and exercise the student's mathematical and numerical abilities while probing the depth of his or her understanding. One sequence of problems works through the derivation of the electromagnetic fields associated with the Lienard-Wiechert potentials, and the vector potential and magnetic moment of a magnetized rotator. The sequence ends with a broad, open-ended exercise on the Crab pulsar, which will prove to be a considerable task for those who follow up the suggested literature search.

But that level of commitment is, I suspect, just what Shu expects. Students who opt to follow this pair of excellent texts are going to equip themselves with a strong background in the physics of astrophysics, and receive a compelling invitation to use this new-found knowledge to explore the many exciting areas of modern astronomy. □

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Fly in the face of reason

Gerald M. Rubin

The Making of a Fly: The Genetics of Animal Design. By Peter A. Lawrence. Blackwell Scientific: 1992. Pp. 228. £16.95, \$29.95 (pbk).

MANY general lessons of developmental biology and pattern formation have emerged from studies of the fruitfly *Drosophila* in the past decade. Fundamental molecular mechanisms are now understood, at least in broad outline. For example, the way in which simple gradients divide the fruitfly embryo into a series of stripes, each only a few cells wide, can now be discussed in terms of the interactions of identified protein molecules and the sites on DNA to which these molecules bind. Our understanding of these processes is arguably one of the great scientific achievements of the twentieth century.

In *The Making of a Fly*, Peter Lawrence attempts to convey the excitement of the field, as well as its history. It is a landmark book in that it is the first and only attempt to bring all this information together in one volume and in a form accessible to students. I would recommend it for any graduate student, post-doctoral researcher or professor in the fields of development, cell biology or genetics — it is pitched at the perfect level for me. Although too difficult for use in an undergraduate course in the United States, the book would be a good choice for first year graduate students,

particularly if it were supplemented with original papers.

Lawrence says in his preface: "The sum of useful information on *Drosophila* would not fit into a hundred books of this size." This is almost certainly true. Perhaps this book would have been more accessible to a larger audience if it had left more to the other 99. This would have made it more analogous to Mark Ptashne's book *A Genetic Switch*, on which it was supposedly modelled. Lawrence could have conveyed a few of the basic concepts more clearly by focusing on one or two examples, say the dividing of the embryo into segments. Instead, he plumped for a more inclusive treatment. The result is a book with too many facts, too many moving parts, too much detail on too many subjects. One is reminded of Ambrose Bierce's trenchant observation: "The covers of this book are too far apart." It is not so much that the covers are too far apart, as that the material between them is too densely packed for an undergraduate text. Leavening is provided by such gratuitous details as a description of Sydney Brenner's eyebrows (page 92, so you don't have to hunt). But for graduate students and researchers interested in developmental biology, the book contains much thought-provoking material and provides a superb way to become exposed to a vast body of information.

Lawrence's views of experimental approaches and the intellectual history of the field keep the book from becoming too dry. He is well suited to the task of writing the book, for he brings a unique and refreshingly broad perspective to the field. He is perceptive, critical and opinionated, although opinion and fact are clearly distinguishable throughout. He writes well. One may disagree with him at times, but his ideas are always well thought out.

The book ends with a series of historical anecdotes. These are highly entertaining and serve well to convey both the excitement of the field and the ways in which ideas develop and mature. The many illustrations are clear and of excellent quality. The book is nicely organized and visually very pleasing. The production is superb.

Lawrence has taken on the extremely difficult, perhaps impossible, task of describing an immense body of information in a simple, concise and comprehensible way. Given the complexity of the task, he has achieved a great measure of success. *The Making of a Fly* fills a huge void and will be sure to inspire a generation of developmental biologists. □

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Texan flying mammals

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The Bats of Texas. By David J. Schmidly. Texas A & M University Press: 1991. Pp. 188. \$34.50 (hbk), \$19.95 (pbk).

TEXAS is the home state of Dr Charles Campbell, whose efforts to demonstrate the value of bats in insect control led to one of the earliest pieces of legislation protecting bats, and the largest bat colonies in the world. In North America, where bats hold a largely misplaced high profile in discussions on rabies, public enthusiasm for these mammals has perhaps been slow to develop. *The Bats of Texas* appears when interest is definitely on the increase; it aims to further this trend and to provide a better understanding of the role of bats in the biological community and the need for their conservation. A general introduction with special reference to the Texan fauna offers that background.

With 32 species in four families, the Texas bat fauna is the most diverse in North America. The fauna includes 29 insectivorous species, two nectarivorous and — recorded only once — the rarer vampire bats. Illustrated keys to these species are given for external characters and skulls. The core of the book consists of accounts of each species, including a brief description and notes on distribution, subspecies, life history, specimens examined and references. A bibliography of 428 references is included. Each species is illustrated with a good-quality black-and-white photograph and county distribution maps (half the species are also shown in colour). The distribution of species is often related to the ecological zones identified within the state, but unfortunately there is little about the special features of the Texan fauna, such as the influence of the nearby tropics and the meeting in Texas of a large proportion of the world's species of the hairy-tailed bats *Lasiurus*.

This is a good and thoroughly researched regional guide. The keys seem simple to use (perhaps too simple for the more difficult genus of the mouse-eared bats *Myotis*) and the species accounts are concise (if sometimes rather overwhelmed by the list of specimens examined). It will certainly appeal to the amateur and professional mammalogist and to many others interested in faunistics and conservation, but it may not open the gate to the wider public. □

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New Journals Issue

This year, *Nature's* annual New Journals review supplement will appear in the issue of 1 October. Publishers and learned societies are invited to submit journals for review, taking note of the following criteria:

■ Journals that first appeared during or after June 1990 and issued at least four separate numbers by the end of April 1992 will be considered.

■ Journals covering any aspect of science are eligible, although those dealing with clinical medicine, engineering and pure mathematics are excluded, as are publications of abstracts.

■ Frequency of publication must be at least three times a year. The main language used must be English. Translation journals in English are, of course, eligible.

■ Deadline for submission is the end of May.

When submitting journals for review, please send at least four different issues (the first, the most recent and any two others) of each title, together with full details of subscription rates (personal and institutional) and frequency of publication, to: Peter Tallack, *Nature*, 4 Little Essex Street, London WC2R 3LF, UK. For further information please telephone Peter Tallack on 071-836-6633 (011-44-71-836-6633 from the United States), extension 2414.

Management decisions

C. M. Perrins

The Balance of Nature? Ecological Issues in the Conservation of Species and Communities. By Stuart L. Pimm. University of Chicago Press: 1992. Pp. 448. \$62, £49.50 (hbk); \$26.95, £21.50 (pbk).

CONSERVATIONISTS are increasingly taking decisions about how best to protect this species or that habitat. Only recently has there been a consensus that it is usually more beneficial to conserve whole communities rather than just specific species (it is still easier to raise money for a showy, endangered bird or mammal than for its habitat). But where does the manager go for advice? And how good is the information available from ecologists?

This book is about communities and their characteristics. Stuart Pimm provides a useful overview of the current state of knowledge about communities, dealing with their stability, resilience (the rate at which they recover their former state after some disturbance), persistence (how long a population remains around one level before changing to another) and resistance to change. His general thesis is that "ecologists have given relatively little attention to long-term, large scale processes, and that, when they do, the importance of multi-species dynamics becomes increasingly important".

Pimm reviews the now copious data on communities and also the large range of theories, often backed up by computer modelling, predicting what communities may do under different circumstances. Particularly instructive are those occasions when the ecologist has experimentally (or man inadvertently) altered a community in some way. A species may be added to, or lost from, a

community and the repercussions of this can be examined. To put it mildly, the results may not always be anticipated.

The trouble for conservationists and managers is that the ecologists' studies often do not yield clear, consistent results that can safely be used as a basis for management programmes for different communities. For example, it might seem obvious that the removal of a predator would lead to an increase in the numbers of a species on which that predator preyed. In practice, however, this may well not be the case; indeed, removal of the predator may even lead to a decrease. This counterintuitive outcome may arise if there are two (or more) competing prey; the removal of the predator may lead to an increase in one of the prey species which will increase the effects of its competition on the other, to the latter's detriment.

An impressive amount of thought and study has been devoted to such issues over the past 15–20 years. But from time to time one is brought up short by the gap between observations and nature. Take Pimm's analyses of the success of man-made introductions of new species. Only some 15 per cent of attempted game-bird introductions have been successful and upwards of 75 individuals need to be released to obtain that level of success. This is enough to put off almost anyone who might wish to attempt such introductions. For the most part, this is probably a good thing, but what of the conservation projects in which attempts are made to reintroduce a species into the wild? Are their chances of success as low as this? If only small numbers of a species are available, is it worth trying at all? Nevertheless, most natural introductions of plants and animals to new habitats (such as islands) presumably involve very few individuals, perhaps only a single pair. Of course, we have no idea of the success rate, but populations obviously can flourish from such modest beginnings. (The same worries apply to theories about the minimum population size needed to maintain genetic diversity.)

The Balance of Nature? will be of most use to ecologists, although I must confess that it left even me feeling a bit at sea. The subtitle suggests that the book is also intended to be read by conservation managers, but I doubt whether it will reach this audience. Too many of the topics have such conflicting data that the book inevitably becomes complex and lacking in clear proposals. This is a shame, because conservation managers have to act and ecologists can often give them useful advice. □

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Methods books

- *Recombinant DNA Laboratory Manual* by Judith W. Zyskind and Sanford I. Bernstein. Revised edition. Academic, \$29.95 (spiral bound).
- *Techniques in Protein Chemistry III* edited by Ruth Hogue Angeletti. Academic, £30 (spiral bound).
- *Methods in Neurosciences. Vol. 8: Neurotoxins* edited by P. Michael Conn. Academic, \$49.95 (spiral bound).
- *Methods in Molecular Biology. Vol. 10: Immunochemical Protocols* edited by Margaret M. Manson. Humana, \$69.50.
- *Neuromethods. Vol. 20: Intracellular Messengers* edited by Alan A. Boulton, Glen B. Baker and Colin W. Taylor. Humana, \$99.50.
- *Quantitative Methods in Neuroanatomy* edited by Michael G. Stewart. Wiley, £60, \$128.
- *Analytical Techniques in Immunochemistry* by Terry M. Phillips. Dekker, \$125.
- *The Baculovirus Expression System: A Laboratory Guide* by L. A. King and R. D. Possee. Chapman and Hall, £30.
- *Plant Tissue Culture Manual* edited by K. Lindsey. Kluwer, \$76.50, £45.50.
- *Molecular Genetic Analysis of Populations: A Practical Approach* edited by A. R. Hoelzel. IRL/Oxford University Press, £22.50 (pbk).

New in paperback

- *The Eclipse of Darwinism: Anti-Darwinian Evolutionary Theories in the Decades around 1900* by Peter J. Bowler. Johns Hopkins University Press, \$13.95, £10. Reviewed by David L. Hull in *Nature* **306**, 714 (1983).
- *The Creative Mind* by Margaret Boden, now with a brief foreward. Cardinal, £6.99. Reviewed by Douglas Hofstadter in *Nature* **349**, 378 (1992).
- *A Dictionary of Plant Pathology* by Paul Holliday. Cambridge University Press, £14.95, \$24.95.
- *Megaherbivores: The Influence of Very Large Body Size on Ecology* by R. N. Owen-Smith. Cambridge University Press, £19.95, \$34.95. For a review see *Nature* **338**, 386 (1989).
- *The Human Psyche: The Gifford Lectures 1978–9* by John C. Eccles. Routledge, £10.99. For a review see *Nature* **292**, 471 (1981).
- *Conceptual Issues in Psychology*, 2nd edn, by Elizabeth R. Valentine. Routledge, £12.99. The first edition was reviewed by P. E. Bryant in *Nature* **302**, 180 (1983).
- *The Social Impact of Computers* by Richard S. Rosenberg. Academic, \$39.95.
- *The Global Environmental Movement* by John McCormick, now with an updated introduction. Belhaven, £14.99. Reviewed in *Nature* **343**, 29 (1990).
- *Supersymmetry and Supergravity*, 2nd edn, by J. Wess and J. Bagger. Princeton University Press, \$22.50, £17. In a review of the first edition, Abdus Salam wrote that "serious students of particle physics would do well to acquire a copy" (*Nature* **307**, 297, 1984).

Other recent ecology books

- *Aquatic Insect Ecology. Vol. 1: Biology and Habitat* by J. V. Ward. Published by Wiley, price £70.
- *Fundamentals of Aquatic Ecology* edited by R. S. K. Barnes and K. H. Mann. Published by Blackwell Scientific, price £18.50 (pbk). For a review of this book's predecessor, see *Nature* **289**, 709 (1981).
- *Ecology: Principles and Applications* by J. L. Chapman and M. J. Reiss. Cambridge University Press, price £15.95 (pbk).
- *British Plant Communities. Vol. 2: Mires and Heaths* edited by J. S. Rodwell. Cambridge University Press, price £95, \$195. For a review of volume 1 on woodlands and scrub see *Nature* **353**, 224 (1991).

Constraints on the age and duration of the last interglacial period and on sea-level variations

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The relation between height and age of shorelines formed during the last interglacial period, as revealed by coral reefs, cannot be related directly to changes in ocean volume because of the effect of isostatic uplift in response to changes in ice-sheet loading. Sea-level changes at sites near the melting ice sheet, such as Bermuda and the Caribbean islands, differ from those along the Australian margin. Modelling of these differences constrains the times of onset and termination of the last interglacial, which are at variance with those deduced from oxygen-isotope studies of deep-sea cores.

THE existence of elevated coral reefs and other marine shorelines of last interglacial age at many locations around the world¹⁻³ has generally been interpreted as evidence for a larger ocean volume during oxygen isotope substage 5e (refs 4, 5) than today⁶. This evidence is not without ambiguity, because shoreline elevations cannot be directly equated with ocean volumes unless the glacio-hydro-isostatic rebound effects are first evaluated. Also, because of these rebound effects, the ages of formation of the shorelines do not provide a direct measure of the age and duration of the substage. As for the Late Holocene sea-level models and observations^{7,8}, the last interglacial levels can be expected to vary not only with time but also with location, depending on position relative to the former ice sheets. Height-age relations of Late Interglacial shorelines along the Australian margin are therefore predicted to be distinctly different from those for locations nearer to the former ice load, such as Bermuda and the Caribbean islands, and these differences can be used to constrain the times of onset and termination of the 5e substage as well as the ocean volume at that time.

When compared with late interglacial sea-level observations from these two regions, the glacio-hydro-isostatic models lead to the conclusions that (1) ocean volumes during the oxygen isotope substage 5e did not exceed their Late Holocene volume, (2) these volumes had been reached by ~135 kyr BP and possibly earlier and (3) the volumes remained at this level until at least 120 kyr BP.

Shoreline evidence

Figure 1 summarizes the observed height-age relations of 5e shorelines for two regions: the tectonically stable margins of Western Australia far from the former ice sheets, and the islands of the Caribbean and Bermuda within the sphere of rebound influence of the former North American ice sheet. The Western Australian observations are from three locations where stratigraphic relationships between sea level and *in situ* corals have been established. The results for both Pelsaert⁹ and Rottne¹⁰ represent lower limits, whereas the Cape Cuvier result¹¹ represents a measure of the height range occupied by sea level during substage 5e. All heights refer to mean low water, the upper limit of present coral growth. The age determinations at the three localities are based on conventional uranium-series dating methods, and the precisions are relatively low. The Cape Cuvier result, including the Lake MacLeod and Cape Range localities, is based on the weighted mean of 11 samples from five different reefs whose individual ages range from 116 ± 6 to 138 ± 7 kyr BP. For all reefs the results are based on a small number of dated samples, and the ages therefore represent a lower limit estimate of the interval in which sea levels were near or above their present level. Taken together, the Western Australian continental margin observations suggest that sea

levels reached their present level by about 135 kyr BP and possibly earlier, and that they remained there until at least 120 kyr BP and possibly as late as 115 kyr BP.

The Caribbean observations illustrated in Fig. 1b are from islands that are believed to be tectonically stable. They include the results from two Bahaman islands, San Salvador and Great Inagua, for which a high-precision uranium-thorium age control is available¹²; from the Cayman Islands where the result represents a lower limit¹³; from the Yucatan Peninsula, where the result is based on samples from several locations¹⁴; from the 'Pleistocene tide gauge' island of Bermuda¹⁵; from northern Bahaman islands on the Great and Little Bahama Banks¹⁶; and from the Lesser Antilles islands of Curacao^{17,18} and La Blanquilla¹⁷ which lie furthest from the former ice sheet. The weighted mean ages for all except San Salvador and Great Inagua are illustrated in Fig. 1b (with weights inversely proportional to the published age variance). The Curacao observation is based on conventionally dated samples from five locations¹⁷ and mass-spectrometrically dated samples from three of the same sites¹⁸. Individual ages range from 141 ± 8 to 123 ± 7 kyr BP. Except for the Lesser Antilles results, the observations indicate shorelines that lie a few metres above the present levels, but the height uncertainties are large. The ages of these reefs all fall within a time interval of ~132–120 kyr BP although, with the exception of the San Salvador and Great Inagua results, the ages are of low precision.

Evidence from rapidly uplifting coastlines is also helpful in defining the interval in which sea levels were near or at their present value. Conventional and high-precision mass-spectrometric ages for the Barbados corals, for example, indicate that sea levels here were near their present levels by 130 kyr BP and remained there until ~122 kyr BP¹⁹⁻²¹; similar results for the raised terraces of western Haiti²² indicate an age range of 132 ± 5 to 122 ± 1 kyr BP. Preliminary high-resolution data from the Huon Peninsula of Papua New Guinea suggests that here the interval may have been longer (~135–120 kyr BP) although not necessarily continuous²³.

With the exception of the mass-spectrometric ages, the ages have low accuracies. Moreover, each reef is represented by only a few samples so that the results may not be representative of the time interval in which the reefs formed. A broader search for the oldest and youngest coral ages in the elevated reefs is desirable for establishing improved constraints on the duration of the interglacial interval.

Glacio-hydro-isostatic rebound models

Figure 2 illustrates the sea-level curve corresponding to the model proposed by Shackleton⁴ from observations of oxygen isotope anomalies in deep-sea cores. These equivalent sea levels $\Delta\zeta_e$ are defined as (change in ocean volume relative to present

volume)/(ocean surface area). According to this model, sea levels reached their present height at ~ 124 kyr BP and fluctuated about this level until 117 kyr BP, attaining a maximum height of +6 m, although a simplified model (I-2) has been adopted in which the high-frequency oscillations in the interval 124–117 kyr BP have been replaced by a zero equivalent sea level. Here we adopt an approximate model for the period before the penultimate glacial maximum at 140 kyr BP. Extensive numerical tests have shown that this model is adequate for describing sea-level change during the interglacial interval. The ice model corresponding to this equivalent sea-level function is similar to the ice-volume changes after 18 kyr BP which were used in previous glacial rebound modelling²⁴. The formulation for the response of the Earth to this ice and associated water load fluctuation has been previously described^{24,25}, and the Earth model adopted is one that has been found to give a good description of Holocene sea-level change for the same regions²⁶. This model is characterized by density and elastic moduli profiles consistent with seismic models, with a 100-km-thick high-viscosity lithosphere, an upper-mantle viscosity of 3×10^{20} Pa s down to a depth of 670 km and a lower-mantle viscosity of 10^{22} Pa s, but a wide range of plausible models give similar results.

Along the continental margin of Western Australia, far from the former ice sheets, highstands of 4–6 m amplitude are predicted to occur early in the interglacial interval (Fig. 3a) because of the adjustment of the Earth to the redistribution of surface loads that occurred immediately before substage 5e. Generally, the predicted amplitudes are comparable to the reported values, demonstrating that observed Late Interglacial highstands do not

necessarily imply that the ocean volume at that time exceeded the present volume, but that they may be, just as the Holocene highstands are, a consequence of glacio-hydro-isostatic rebound. Like the far-field Holocene highstands, the amplitudes of these interglacial highstands vary along the coastline, in part because of ocean geometry and distance of the site from the open sea, and in part because of different distances from the ice sheets. This is illustrated in Fig. 3a for the offshore Rottneest Island, the coastal Cape Cuvier site and the site of Lake MacLeod now 70 km inland. For most plausible combinations of Earth and ice models, however, this variation is predicted to be ~ 1.5 –2.0 m and is unresolvable with the available data.

Figure 3b illustrates predictions for three intermediate-field locations: Bermuda, Barbados and San Salvador (Bahamas). At none of these sites does the ice model I-2 produce significant highstands, because the glacio-hydro-isostatic deformation here is dominated by the glacial unloading, which is negative in this region, with the result that sea levels are predicted to lie below their present value. The predictions do indicate that at all sites the highest levels occur at the end of the interglacial interval, when the glacial unloading contribution has partly relaxed and before the lowering of sea level by renewed glaciation becomes significant. The predicted absence of a highstand is most pronounced at Bermuda where no plausible Earth model used in conjunction with the ice model I-2 produces a highstand similar to that observed (Fig. 1b). Significant tectonic uplift of this island is improbable, for if any vertical movement has occurred in the past 100 kyr, it is likely to have been one of slow subsidence²⁷. If the ocean volumes during substage 5e are increased in the model so as to raise equivalent sea level by 5–7 m, then the shorelines in the Caribbean and Bermuda approach the observed levels but the predicted far-field highstands become excessively high. Alternatively, higher interglacial sea levels can be achieved if the duration of the 5e interval is lengthened so that there is greater relaxation of the glacial unloading effect before ocean volumes again decrease. Thus the amplitude of substage 5e highstand at these intermediate-field locations is a function of both the ocean volume and the duration of the substage, with the maximum elevations occurring late in the interglacial interval. Both the San Salvador and Great Inagua observations support the predicted trend of a rising sea level during substage 5e (Fig. 1b). In contrast, at the far-field continental margin sites the amplitude is primarily a function of the ocean volume during this stage with the maximum highstand occurring early in the interglacial interval.

The high-precision observations of reef age from San Salvador¹² establish an upper limit of ~ 120 kyr BP for the end of the interglacial stage. This is consistent with precise data from Bermuda¹⁵ and other locations throughout the region,

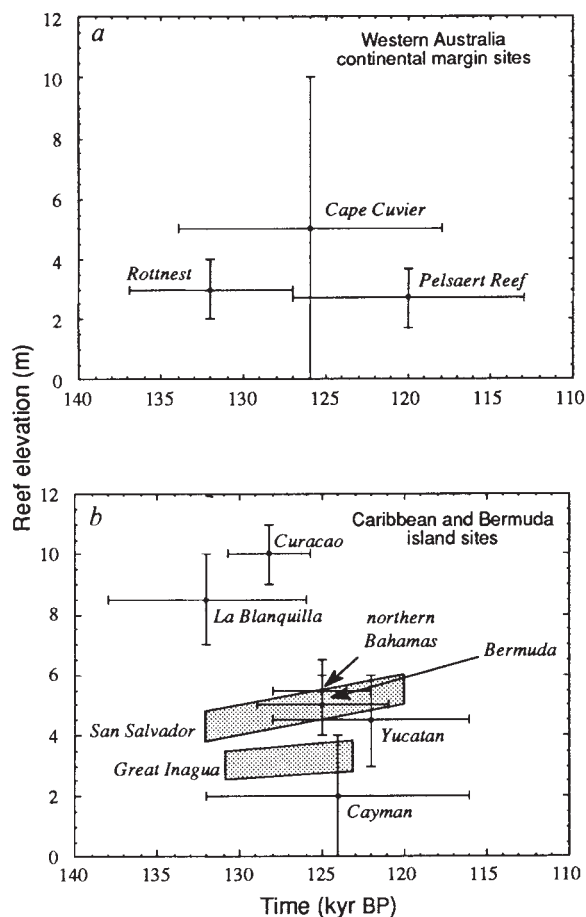


FIG. 1 Observed height–age relationships of elevated reefs along (a) the continental margin of Western Australia, and (b) at tectonically stable sites in the Caribbean and northwest Atlantic (see text for references).

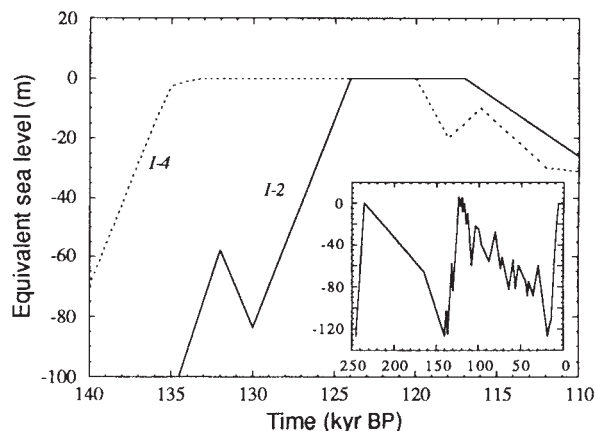


FIG. 2 Equivalent sea-level function for the ice models I-2 and I-4 at the time of oxygen isotope substage 5e and (inset) the Shackleton⁴ curve for the interval 140–0 kyr BP and the approximate function from 250 to 140 kyr BP.

suggesting that the end of the interglacial interval in the adopted ice models should be shifted back in time by ~ 3 – 5 kyr from that given by the Shackleton-based model I-2. For these models, moreover, the interval in which sea levels at both Bermuda and the Bahaman islands are predicted to lie near or above their present level is less than the duration of the interglacial interval itself, and the local sea-level response lags the equivalent sea-level function by ~ 5 – 7 kyr (compare Fig. 3b with Fig. 2). The presence of raised coral reefs on San Salvador by 132 kyr BP therefore implies that equivalent sea level approached its present level considerably earlier, possibly by ~ 139 – 137 kyr BP.

Comparison with observations

In the ice model I-4 (Fig. 2) the ocean volume is assumed to have reached its present value at 135 kyr BP and remained at this level until 120 kyr BP. Sea-level changes before 135 kyr BP are the same as in model I-2 except that all ages have been translated by 11 kyr. Melting of the earlier ice sheet did not cease abruptly as in model I-2, and the final few metres of equivalent sea level are assumed to have been added over a period from 135 to 133 kyr BP, similar to that proposed for the final decay of the last ice sheet in mid-Holocene time²⁸. From the evidence of sea levels at San Salvador and Great Inagua near or just above the present level as early as 132 kyr BP, the model I-4 must be considered to be a minimum duration model for substage 5e. Figure 4 illustrates the corresponding predicted sea levels for the two regions. The maximum highstand amplitudes reached in the far-field at Rottnest (Fig. 4a) are typically 3–5 m for a range of plausible Earth models. The oldest corals above present sea level are predicted to be ~ 135 kyr BP, and corals at or above this level can be expected to span an age

range of 135–120 kyr BP. Similar results are predicted for all far-field sites including ocean islands.

The predictions for the intermediate field sites in the Caribbean and Bermuda are illustrated in Fig. 4b. Now, other than for the more distant sites of Barbados or the Lesser Antilles, the local sea levels do not reach their present value until ~ 127 kyr BP, after which, and until the end of substage 5e, sea levels increase with time. The maximum attained amplitudes of the highstands are predicted to increase from north to south. The predictions are generally consistent with the observed trends of substage 5e observations across the region. The only large discrepancy with observation occurs for the Lesser Antilles islands of Curacao and La Blanquilla for which the predictions are similar to those for Barbados and where no Earth model predicts the observed highstands of 7–10 m (ref. 17). Thus a tectonic uplift of 0.05 – 0.10 mm yr⁻¹ must be assumed to have occurred if the observations are correct.

Implications

On the basis of these comparisons of the observed sea levels in substage 5e with the models of glacio-hydro-isostatic rebound, our conclusions are that the ocean volume during this substage was similar to the present volume, and that the substage started by or before 135 kyr BP and lasted until at least 120 kyr BP. Both conclusions are relevant to important issues in Quaternary science. For example, several terrestrial, marine or glaciological indicators^{5,29–31} indicate that regional warming may have occurred during the last interglacial relative to the Late Holocene, although evidence for global differences between the two periods remains scant⁵. If, as often assumed, ocean volumes were greater during substage 5e than during the Late Holocene, this suggests

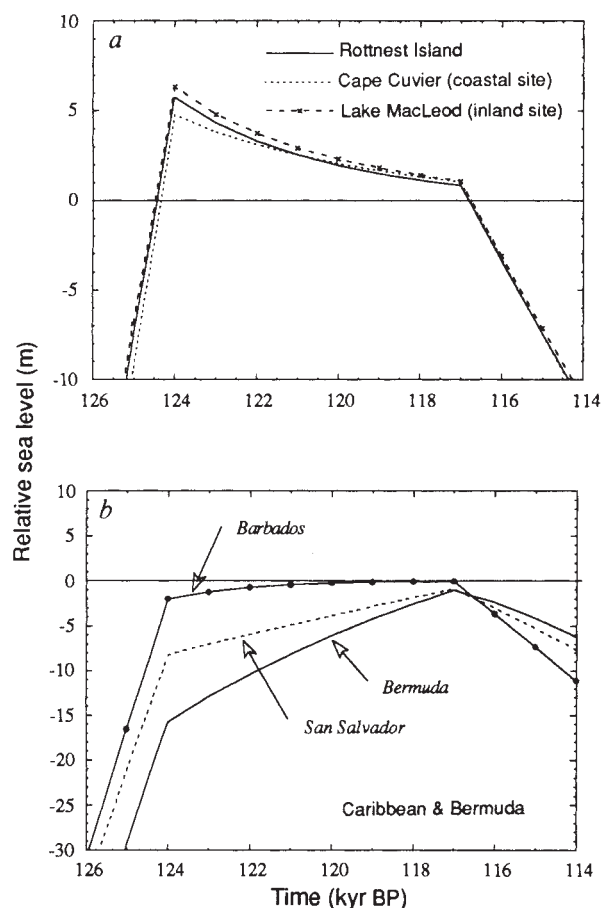


FIG. 3 Predicted sea levels based on the ice model I-2 for (a) sites along the Eastern Australian margin, and (b) sites in the Caribbean region and Bermuda.

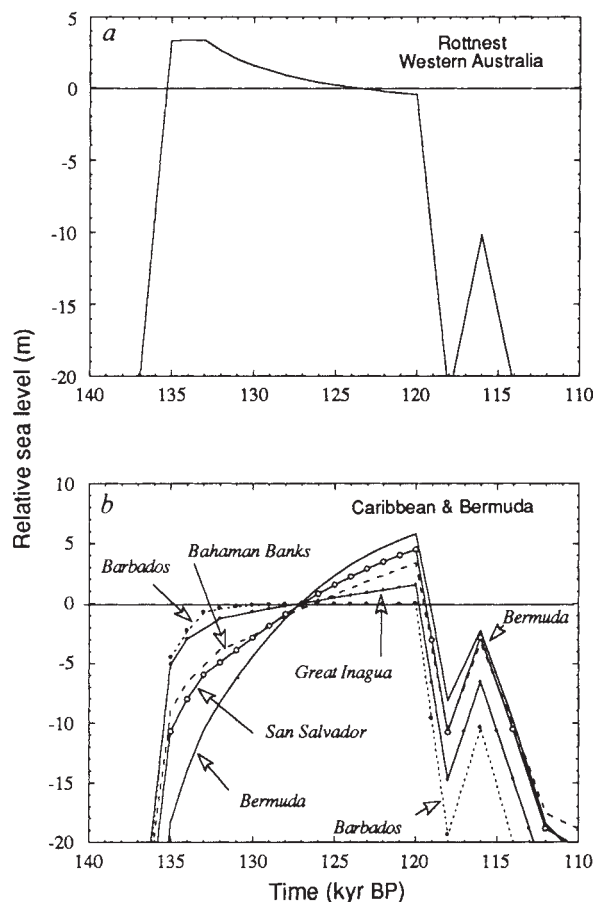


FIG. 4 Predicted sea levels based on the ice model I-4 for (a) Rottnest Island, and (b) Caribbean islands and Bermuda.

that the remaining ice sheets (such as that of the West Antarctic) must be sensitive to minor climate warming. But the models for the glacio-hydro-isostatic adjustment of the Earth remove the principal argument for larger ocean volumes at that time, so that if the climate was indeed warmer during substage 5e then these residual ice sheets must be relatively stable in the presence of small temperature increases.

Another issue raised is the duration of the interglacial interval. The evidence from raised reefs is that sea levels were near their present level from at least 135 to ~120 kyr BP, an interval longer than the 7–12-kyr interval inferred from the oxygen isotope record from deep-sea cores and scaled by astronomical

motions^{4,5}. It has been usual to ascribe the discrepancy to inaccurate dating of the reefs, but even with recent improvements in uranium-series dating, ages as old as 130–132 kyr BP are still indicated for some Caribbean corals; because of the glacio-hydro-isostatic adjustments of the Earth at these localities, the ocean volumes would have reached their present value earlier than this, possibly by 135–137 kyr BP. Improved age constraints on interglacial reefs at or above present sea level, particularly from better sampling of the individual reefs, coupled to glacio-hydro-isostatic rebound models, should contribute considerably to resolving this apparent conflict of the evidence for the onset and duration of the last interglacial interval. □

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ATP-dependent recognition of eukaryotic origins of DNA replication by a multiprotein complex

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A multiprotein complex that specifically recognizes cellular origins of DNA replication has been identified and purified from the yeast *Saccharomyces cerevisiae*. We observe a strong correlation between origin function and origin recognition by this activity. Interestingly, specific DNA binding by the origin recognition complex is dependent upon the addition of ATP. We propose that the origin recognition complex acts as the initiator protein for *S. cerevisiae* origins of DNA replication.

THE complex process of eukaryotic chromosomal replication must be carefully regulated throughout the cell cycle. It is likely that much of this regulation occurs at the level of the initiation of DNA synthesis. Studies of *Escherichia coli* chromosomal, bacteriophage, and eukaryotic viral DNA replication have resulted in a paradigm for the initiation of bidirectional DNA replication^{1,2}. For each of these organisms, the first step is the sequence-specific recognition of the origin of DNA replication by an initiator protein. Binding of the initiator causes partial untwisting of the double helix adjacent to the recognition site and the subsequent action of a helicase leads to further unwinding of the DNA duplex. This unwound DNA structure serves as a template for the initiation of DNA synthesis. Although protein factors likely to be involved in the unwinding and elongation stages of eukaryotic chromosomal DNA replication have been described^{3,4}, proteins involved in the initial stage of eukaryotic origin recognition have not been identified.

The availability of short well characterized chromosomal

origins of DNA replication derived from the yeast *S. cerevisiae* make it a particularly useful organism to study the earliest steps of eukaryotic DNA replication. Originally identified as chromosomal sequences able to confer high frequency of transformation on plasmid DNA⁵, a subset of these autonomous replication sequences (ARS) were subsequently shown to act as true origins of replication in the chromosome^{6–9}. Sequence comparison of numerous ARS elements led to the identification of an 11-base-pair sequence that is conserved across all ARS elements and is referred to as the ARS core consensus sequence (ACS)^{10,11}. Studies of the sequences required for ARS function have found that although the ACS is necessary, sequences 3' to the T-rich strand of the ACS also are required for ARS function¹¹. A detailed analysis of *ARS1* identified four *cis*-acting elements that constitute this chromosomal origin of DNA replication¹². In addition to an essential A element containing the ACS, three distinct elements within the 3' region, B1, B2 and B3, are required for efficient *ARS1* function. One of these elements, B3, is a

binding site for the yeast DNA-binding protein ABF1 and can be substituted by the binding sites of other transcription factors. The remaining two elements, B1 and B2, have no known function, but seem to be functionally distinct from both each other and the B3 element.

Studies directed at identifying proteins that recognize *S. cerevisiae* origins of replication have identified several proteins that recognize sequences outside the ACS, including ABF1 (refs 11, 13–18). More recently, a single-stranded DNA-binding protein that recognizes the T-rich strand of the ACS has been described^{19–21}. Despite these efforts, no protein has been identified that can recognize the double-stranded form of the ACS. We have used a DNase protection assay to identify a novel DNA-binding activity that specifically recognizes the double-stranded ACS in an ATP-dependent manner. Our findings suggest that this multiprotein complex acts as an initiator protein at chromosomal origins of DNA replication.

Identification and purification of ORC

Initial DNA binding studies using crude yeast nuclear extract and *ARS1* DNA detected only the abundant nuclear protein ABF1. Fractionation of this extract, however, revealed a distinct DNA binding activity. This activity protected both the A and B1 elements of *ARS1* from deoxyribonuclease I digestion. Moreover, this binding event showed a strong dependence on the addition of exogenous nucleoside triphosphates. Through the use of both conventional and DNA-affinity chromatography,

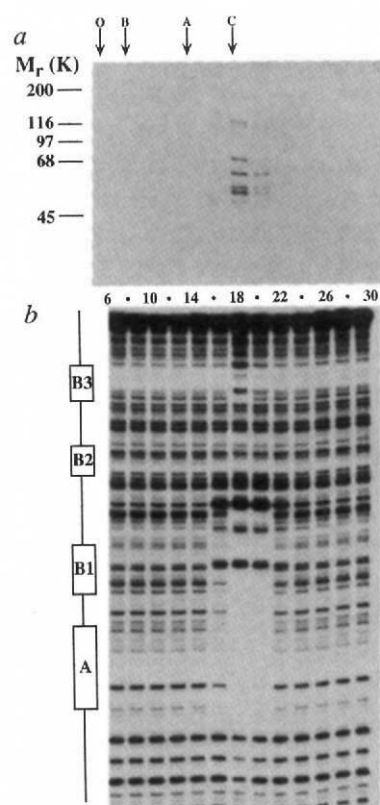
this DNA-binding activity was purified to near homogeneity. Fractionation of the binding activity by glycerol gradient sedimentation indicated that it had a relative molecular mass of ~250,000 (M_r 250K). SDS-polyacrylamide gel analysis of fractions collected from the final glycerol gradient of the purified activity showed no proteins that could individually account for such a large size. Instead, eight polypeptides of M_r 120K, 116K, 97K, 72K, 62K, 56K, 53K and 50K co-sedimented with the DNA-binding activity (Fig. 1). Unless otherwise indicated, this material was used for all subsequent experiments. The abundance of the 120K, 116K and 97K polypeptides varied significantly between different preparations, suggesting that the 116K and 97K proteins are proteolytic products of the 120K polypeptide. Additional chromatographic steps were unable to separate this set of proteins. Together these findings suggest that a multiprotein complex is responsible for the origin binding activity. We will refer to this DNA-binding activity as the origin recognition complex (ORC).

ORC was biochemically unrelated to the previously identified factor(s) that recognize the single-stranded form of the ACS (refs 19–21). ORC binding was not reduced by the addition of up to 10,000-fold molar excess of the T-rich strand of the ACS. In addition, purified preparations of ORC were unable to alter the mobility of either single-stranded or double-stranded DNA fragments containing the ACS in non-denaturing polyacrylamide gels (data not shown).

Titration of ORC protein into the footprinting assay revealed

FIG. 1 Purification of ORC. **a**, Glycerol gradient sedimentation of ORC. Aliquots (40 μ l) of every second fraction from the final glycerol gradient were precipitated with trichloroacetic acid, dissolved in SDS sample buffer and analysed on a 10% SDS-polyacrylamide gel. Proteins on the gel were stained with silver. The fractions tested are indicated below. The position of the SDS-PAGE M_r size marker proteins are shown on the left. The position of protein markers sedimented in a parallel glycerol gradient are indicated above the gel: O, ovalbumin; B, bovine serum albumin; A, aldolase; C, catalase. **b**, Sedimentation of *ARS1* DNA-binding activity. DNase protection reactions were performed on the T-rich strand of the ACS of *ARS1* prepared as described in the legend to Fig. 2. Every second fraction (0.6 μ l) collected from the final glycerol gradient was tested (indicated above each lane). The positions of the elements of *ARS1* are shown on the left¹². No protein or DNA-binding activity was detected in fractions 1–5.

METHODS. Nuclear extract was prepared from 145 g (wet weight) of logarithmically growing BJ926 cells. All buffers contained 0.5mM PMSF, 1 mM benzamide, 2 μ M pepstatin A, 0.1 mg ml⁻¹ bacitracin and 2 mM dithiothreitol. Nuclear extracts were prepared as described⁴³ with the following changes. Spheroplasts were washed by loading onto a 150-ml cushion of 0.8 M sucrose, 1.5% Ficoll and 20 mM Tris-HCl buffer, pH 7.5, in a 250-ml conical bottle. The spheroplasts were spun through the cushion at 3,000g for 10 min. Spheroplasts were lysed by a single pass through a Yamato LH-21 homogenizer. After dialysis against buffer H/0.1 (50 mM HEPES-KOH, pH 7.5, 0.1 M KCl, 1 mM EDTA, 1 mM EGTA, 5 mM magnesium acetate, 0.02% NP40, 10% glycerol) the nuclear extract was applied to an S-Sepharose column (Pharmacia-LKB) equilibrated with H/0.1 (2.5 $\times$ 9 cm, 15 mg protein per ml resin). The column was washed with buffer H/0.2 (the above buffer with 0.2 M KCl) and developed with a 500-ml linear gradient of KCl (0.2–0.6 M) in buffer H. The active fractions (0.38 M KCl) were pooled and dialysed against T/0.18 (25 mM Tris-HCl, pH 8.0, 0.18 M KCl, 1 mM EDTA, 1 mM EGTA, 5 mM magnesium acetate, 0.02% NP40, 1 mM DTT, 10% glycerol). Proteins were then loaded on to a Q-Sepharose column equilibrated in T/0.18 (1.5 $\times$ 3 cm, 6 mg protein per ml resin) and eluted with a 60-ml linear gradient of KCl (0.18–0.6 M) in buffer T. The active fractions (0.3 M KCl) were dialysed against buffer H/0.1. The dialysed pool was loaded onto a double-stranded DNA-cellulose column (Sigma) equilibrated in H/0.1 (0.3 $\times$ 3.5 cm, 5 mg protein per ml resin) and eluted by a linear gradient of KCl (0.1–0.6 M) in buffer H. The active fractions (0.2M KCl) were dialysed against H/0.1. The dialysed pool (2.6 mg) was brought to 0.5 mM ATP, 5 mM CaCl₂ and 10 mM MgCl₂ and loaded onto a sequence-specific DNA affinity column equilibrated in buffer H⁺/0.1 (H/0.1 containing 0.5 mM ATP, 5 mM CaCl₂, and 10 mM MgCl₂) and prepared as described⁴⁴ using the oligonucleotides 5'-GATCT-AAACATAAAATCTGTAAAA-3' and 5'-GATCTTTTACAGATTTTATGTTA-3'. Bound proteins were eluted with a linear gradient of KCl (0.1–0.6M) in buffer H⁺. The active fractions (0.2M KCl) were dialysed against H/0.1. Proteins



were concentrated on a 200- μ l double-stranded DNA-cellulose column equilibrated in H/0.1 followed by a step elution with H/0.5. The peak fractions were applied to a 5-ml, 15–35% glycerol gradient in buffer H/0.15 and spun at 225,000g for 12 h. Fractions (200 μ l) were collected from the bottom of the gradient. The two peak fractions were concentrated on double-stranded DNA-cellulose and applied to a second glycerol gradient prepared and sedimented as before. Fractions (175 μ l) were collected from the top of the gradient. This procedure yielded ~10 μ g purified ORC protein. A footprinting assay was used to detect ORC activity throughout the preparation. Footprinting reactions are described in Fig. 2 legend.

several interesting features of this DNA-binding event (Fig. 2, lanes 1–7). At low concentrations of ORC protein, three sites of enhanced DNase I cleavage were observed, one within element B1 and two between elements B1 and B2 of *ARS1* (lane 2). Increasing amounts of ORC protein protected a 50-base-pair region that included elements A and B1 and enhanced DNase cleavage at two additional sites between elements B1 and B2 (lanes 3 and 4). Binding of ORC to the A-rich strand of *ARS1* showed a similar DNase footprint, but the sites of enhanced cleavage were offset two to three base pairs towards the ACS (Fig. 2, lanes 8–10). The observed 10-base-pair periodicity of DNase cleavage on both strands of *ARS1* suggests that the DNA may wrap around ORC during binding²². At higher concentrations there were new regions of protection, including weak protection of the B2 element and a second region of alternating protection and enhanced cleavage with a 10-base-pair periodicity encompassing the B3 region of *ARS1* (Fig. 2, Lanes 5 and 6).

Specificity of ORC DNA binding

The DNase protection pattern produced by ORC binding encompassed two DNA sequence elements that are critical for *ARS1* function. To address whether either of these elements are recognized by ORC, several linker scanning mutants in *ARS1* (ref. 12) were tested directly for ORC binding (Fig. 3a). An *ARS1* mutant simultaneously defective in B1, B2 and B3 function (B123-KO) showed essentially the same DNase protection pattern as wild-type *ARS1*, indicating that the B elements were

not required for specific recognition (lanes 9–11). Similarly, a linker scanning mutant located between elements A and B1 that did not affect *ARS1* function¹², had no effect on ORC binding (lanes 3 and 4). In contrast, two mutants that disrupt the A element and prevent *ARS1* function (*ARS1*/858–865 and *ARS1*/865–872)¹² both dramatically altered the DNase protection pattern observed in the presence of ORC protein (lanes 5–8). Unlike the wild-type situation, no protection was observed over the A or B1 elements. Instead, there was a new region of protection encompassing the B2 element. The B2 element of *ARS1* is composed of a 9 out of 11 match to the ACS in the opposite orientation to the exact match located within the A element. The observed protection pattern, which initiates at B2 and extends towards the B1 element, suggested that in the absence of an exact match to the ARS consensus the ORC protein bound to the imperfect ACS at B2. Interestingly, in the absence of a functional A element the protection pattern at B3 is unchanged, suggesting additional interactions with these sequences independent of the A element. These findings, however, suggest that the ACS is the predominant element responsible for ORC DNA binding.

To address more precisely the role of the ACS in ORC DNA binding, seven point mutations were constructed in this sequence at the A element of *ARS1*. All of the mutants showed reduced DNase protection by ORC relative to wild-type *ARS1* DNA, although the extent of this reduction varied (Fig. 3b). Only one mutant, 860T→A, retained significant, albeit reduced, protection of the A element of *ARS1* in the presence of ORC protein. Four other mutants, 859T→A, 859T→C, 860T→C, and 863A→G, all showed minimal, if any, protection of the ARS consensus sequence, but retained the strong enhanced DNase cleavages characteristic of ORC binding and significant protection of a portion of the B1 element (lanes 4–7, 10–11, and 16–17). The most deleterious of the point mutations tested were 860T→G and 863A→C (lanes 12–15). These mutants showed little or no protection over either the A or B1 elements of *ARS1* and exhibited a significant reduction in the extent of enhanced DNase cleavage. In addition the point mutants also show weak protection of the B2 element, consistent with the binding observed with the linker scanning mutants within the A element of *ARS1*. Taken together, the results of the mutant DNA binding studies confirm that the ACS is required for the recognition of *ARS1* by ORC.

To correlate the effect of these *ARS1* point mutations on ORC binding with the function of *ARS1* as an autonomous replicating sequence, these same point mutants were tested for their function in a plasmid stability assay. The results of these studies show a strong correlation between ORC binding and plasmid stability. Thus, the mutation that reduced ORC DNA binding the least, 860T→A, retained the ability to transform yeast at high frequency. The plasmid stability of these transformants was about five-fold less than wild-type *ARS1*, consistent with the reduced binding of ORC to this mutant. Of the remaining mutants, only 859T→C transformed yeast at high frequency, and the plasmid stability of these transformants was reduced by at least 50-fold relative to 860T→A, as expected, given the reduced binding of ORC to this mutant. The close correspondence between ORC binding and ARS function exhibited by these mutants supports the hypothesis that ORC both recognizes and functions at the ACS.

ORC binding at other ARS elements

To address whether ORC binds other yeast origins of replication or exclusively *ARS1*, we tested ORC binding to three additional well characterized ARS elements, *ARS307*, *H4ARS* and *ARS121*. Like *ARS1*, *ARS307* and *ARS121* act as chromosomal origins of DNA replication (C. Newlon, personal communication)²³. The *H4ARS* has not been tested for origin function. Strong ORC-dependent DNase protection was observed over the minimal sequences required for the function of the

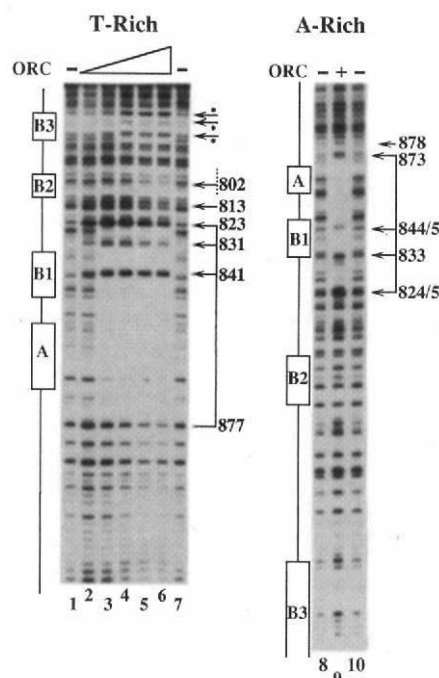
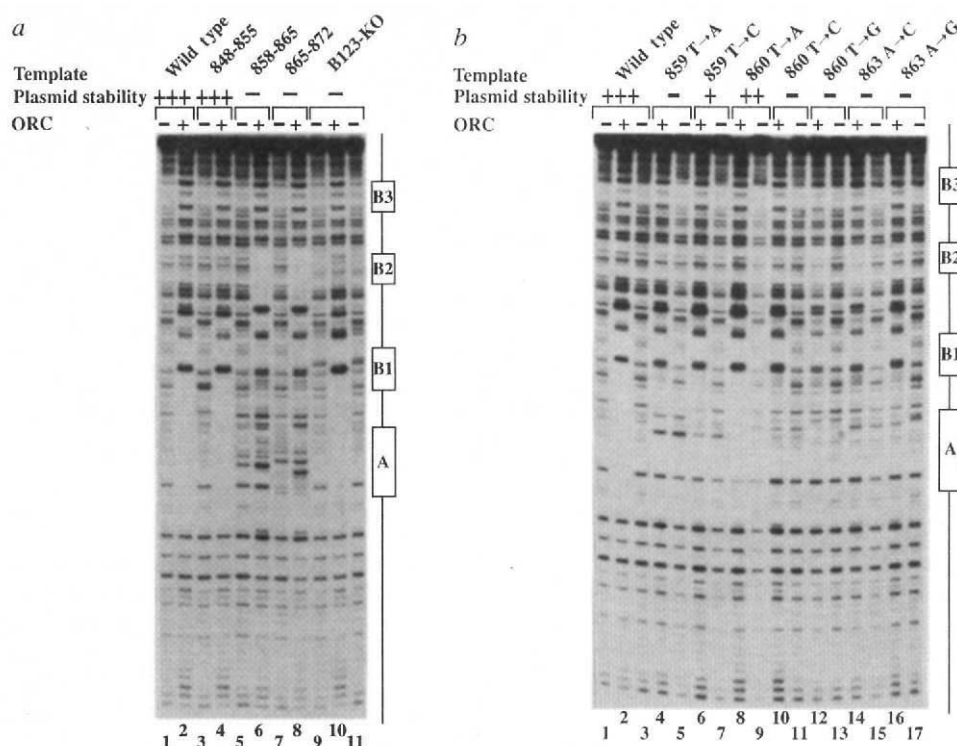


FIG. 2 ORC binds multiple regions of the *ARS1* origin of DNA replication. DNase footprints were done with DNA fragments containing *ARS1* sequences between 734 and 926 (ref. 5) labelled with ³²P at the 5' end of either strand (lanes 1–7, the T-rich strand of the A element labelled; lanes 8–10, the A-rich strand of the A element labelled). Lanes 1, 7, 8 and 10 contain no protein. Lanes 2 to 6 contain 4, 8, 16, 32 and 64 ng ORC respectively. Lane 9, 16 ng ORC. The positions of the elements of *ARS1* are indicated to the left of each footprint. The positions of regions of enhanced or reduced DNase cleavage are indicated to the right of each panel. Brackets and asterisks indicate regions of DNase protection. Arrows indicate sites of enhanced DNase cleavage and the numbers to the right indicate their position.

METHODS. Binding reactions (25 μ l) contained 37.5 mM HEPES-KOH, pH 7.5, 10 mM magnesium acetate, 3 mM EGTA, 0.5 mM EDTA, 200 μ M each of CTP, GTP and UTP, 4 mM ATP, 125 μ M each of dCTP, dGTP and TTP, 25 μ M dATP, 2% polyvinyl alcohol, 5% glycerol, 200 ng poly d(A-C), 40 mM creatine phosphate and 0.06 units of creatine phosphokinase. Binding was at 30 °C for 10 min, followed by standard DNase digestion⁴⁵.

FIG. 3 DNA-binding specificity of ORC. *a*, ORC binding to *ARS1* is disrupted by mutants in the A element. Wild-type or mutant *ARS1* DNA fragments labelled at position 926 and extending to position 734 were used for DNase footprinting (T-rich strand of the A element is labelled). Templates used are indicated above each set of reactions (B123-KO is *ARS1*/756, 758, 802-808, 835-842)¹². Lanes 1, 3, 5, 7, 9 and 11 contain no protein. Lanes 2, 4, 6, 8 and 10 contain 32 ng ORC. The elements of *ARS1* are indicated to the right. The plasmid stability of each construct is indicated above each set of reactions¹². *b*, Point mutants in the *ARS1* ACS reduce ORC binding and *ARS1* function. Wild-type *ARS1* or point mutants in the 11 out of 11 match to the ACS consensus sequence at *ARS1* labelled at position 943 and extending to position 734 were used for DNase footprinting (T-rich strand of the A element is labelled). Templates used are indicated above each set of reactions. Lanes 1, 3, 5, 7, 9, 11, 13, 15 and 17 contain no protein. Lanes 2, 4, 6, 8, 10, 12, 14 and 16 contain 24 ng ORC. The elements of *ARS1* are indicated to the right. The plasmid stability of each construct is indicated above each set of reactions: +++, 30–40%; ++, 6–7%; +, high frequency of transformation-positive, plasmid stability below detection (<0.05%); –, minus for high-frequency of transformation.



METHODS. DNase footprinting was performed as for Fig. 2. High frequency of transformation and plasmid stability assays were as described¹². Point mutants in the *ARS1* ACS were constructed by site-directed mutagenesis.

three ARS elements (Fig. 4). In each case, the region of protection included the essential match to the ACS as determined by previous mutagenesis studies^{24–26}. As with *ARS1*, these protected regions were ~50 base pairs in length and were located asymmetrically with respect to the ACS, extending significantly further in the direction 3' to the T-rich strand of the consensus sequence. The *ARS307* and *H4ARS* footprints also showed similar ORC induced sites of enhanced DNase cleavage as seen in *ARS1*. At each ARS these sites initiate 12–16 base pairs 3' to the T-rich strand of the ACS and are spaced by ~10 base pairs (lanes 1–6), with the exception of one slightly offset site in *ARS307*. The absence of enhanced bands at *ARS121* may be due to the lack of an exact match to the ACS at this origin of DNA replication. Although there are a number of other partial matches to the ACS consensus sequence found in all of these ARS elements, only the essential match is fully protected from DNase digestion. The selective protection of the essential match to the ACS at these three distinct ARS elements indicates that ORC recognizes other origins of replication in addition to *ARS1* and strongly suggests that ORC mediates the function of the ACS at these sequences as well.

Identification of DNA proximal subunits of ORC

To identify the subunits of ORC close to the DNA at the time of binding we crosslinked the complex to radiolabelled DNA. To prepare the DNA for crosslinking, ³²P-labelled dCTP and a photocrosslinking nucleotide (N₃RdUTP; ref. 27) were incorporated at specific sites within the region of *ARS1* protected from DNase digestion by ORC. When these modified *ARS1* DNA fragments were exposed to ultraviolet light in the presence of purified or partially purified ORC, polypeptides within reach of the photoreactive nucleotide were covalently linked to the ³²P-labelled DNA (Fig. 5). Using partially purified preparations of ORC, multiple polypeptides were crosslinked to DNA in this manner (Fig. 5, lane 2). DNA competition experiments using *ARS1* DNA fragments that contained either a wild-type or

mutant ACS identified three polypeptides of *M_r* 72K, 62K and 53K that competed specifically with wild-type but not mutant DNA (compare lanes 3 and 4). Crosslinking of the same set of three polypeptides was reduced significantly when nucleoside triphosphate (ATP) was omitted from the reaction mixture. The remaining polypeptides exhibited enhanced crosslinking, presumably because of increased accessibility to the modified nucleotide (lane 6). When the same crosslinking reaction was done using purified ORC preparations, only four polypeptides were crosslinked, including the three polypeptides identified as sequence-specific by DNA competition experiments (lane 1). These same four polypeptides are abundant in purified ORC preparations and comigrated exactly with the crosslinked adducts when run side-by-side on the same SDS gel (data not shown). The finding that the 120K subunit of ORC is non-specifically crosslinked suggests that either it is a nonspecific DNA-binding protein associated with ORC or a contaminant of the ORC preparation. Although our experiment used a unique site of modification (position 853 in *ARS1*; between B1 and A), similar results were obtained when other sites of modification were used (data not shown). These findings indicate that the 72K, 62K and 53K polypeptides constitute part of the origin recognition complex and are closely juxtaposed to the DNA during binding. Nevertheless, owing to the long reach of the photocrosslinking nucleotide used (9–10 Å)²⁷, it is possible that either one or some combination of the crosslinked polypeptides is responsible for specific recognition of the ACS.

Nucleotide requirement for ORC DNA binding

The unusual requirement for nucleoside triphosphates to allow specific DNA binding by ORC was maintained throughout its purification. To address the specificity of this requirement, footprinting experiments were done in the absence or presence of a number of different nucleotides (Fig. 6). In the absence of added nucleotides, the DNase cleavage pattern was nearly identical to that observed in the absence of added protein (compare

lanes 6 and 7). Addition of ATP alone resulted in a protection pattern that was indistinguishable from that when all eight nucleoside triphosphates were added to ORC footprinting reactions (compare Fig. 6, lane 2 with Fig. 2). None of the remaining three ribonucleoside triphosphates stimulated DNA binding at a level comparable to ATP, although the addition of CTP allowed a low but significant level of ORC binding. At higher concentrations (1mM), all four ribonucleoside triphosphates stimulated ORC DNA binding (data not shown) but the effect of ATP remained significantly stronger than CTP, GTP and UTP. To determine the role of the triphosphates during ORC binding, two ATP analogues were tested for their ability to function in the DNA binding assay. ATP- γ -S stimulated ORC DNA binding at a level comparable to ATP (lane 10). In contrast, AMP-PNP (β - γ -imidoadenosine 5'-phosphate), which unlike ATP- γ -S has a non-hydrolysable β - γ bond, was unable to stimulate ORC DNA binding (lane 11).

The exact role of ATP in ORC DNA binding remains unclear. The inability of AMP-PNP to substitute for ATP during footprinting suggests that hydrolysis of the ATP β - γ bond is required for sequence-specific DNA binding by ORC. These

findings suggest that ORC undergoes a significant change in conformation upon binding, and possibly hydrolysis, of ATP. Such a change could involve an alteration in the ORC DNA binding domain or alternatively a rearrangement of one or more of the ORC subunits. Despite the apparent requirement for ATP hydrolysis, we have yet to detect an ATPase activity in purified ORC preparations. It is also possible that the ATP requirement reflects a protein phosphorylation event, although autophosphorylation has not been detected in purified preparations of ORC either in the presence or absence of DNA.

Discussion

The replicon model²⁸ hypothesized that DNA replication would be controlled by a positive regulator (the initiator) acting on a *cis*-sequence (the replicator). Studies of *E. coli*, bacteriophage and eukaryotic viral DNA replication have largely upheld these predictions. Unlike these simple replication units in which a single bidirectional origin of DNA replication is sufficient to direct all DNA synthesis, eukaryotic chromosomal DNA replication involves coordinated initiation from multiple origins. In yeast, a subset of ARS elements act as origins of replication and the ARS consensus sequence very likely fulfills the role of a replicator. The corresponding initiator protein, however, has

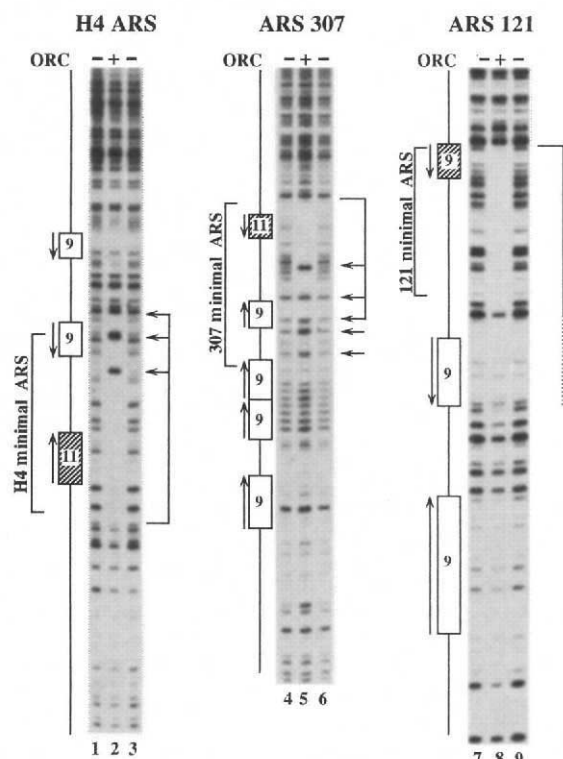


FIG. 4 ORC recognizes essential sequences in three other ARS elements. In lanes 1–3 an *H4* ARS-containing DNA fragment labelled at position 1 and extending to position 174 was used for DNase footprinting (the T-rich strand of the ACS match is labelled; numbering according to ref. 24). In lanes 4–6 an *ARS 307*-containing DNA fragment labelled at position 1 and extending to position 522 was used for DNase footprinting (the A-rich strand of the ACS match was labelled; numbering according to ref. 25). In lanes 7–9 an *ARS 121*-containing DNA fragment labelled at position 486 and extending to position 1 was used for DNase footprinting (the A-rich strand of the essential match to the ACS is labelled; numbering according to ref. 26). Lanes 1, 3, 4, 6, 7 and 9 contain no protein. Lanes 2, 5 and 8 contain 16 ng ORC. Matches to the ACS are shown in boxes to the left of each panel (9, 9 out of 11 match; 11, exact match). The essential match to the ACS for each ARS is shaded^{24–26}. Vertical arrows indicate the 5' to 3' direction of the T-rich strand of the ACS. The horizontal arrows indicate the DNase hypersensitive sites are at positions 126, 116/117 and 107 from top to bottom at *H4* ARS. For *ARS 307* hypersensitive sites are at positions 170, 158, 149, 146 and 139 from top to bottom. Bracketed regions to the left of each panel indicate the minimal regions of each ARS required for high frequency of transformation^{23–25}. Bracketed regions to the right of each panel indicate regions of DNase protection.

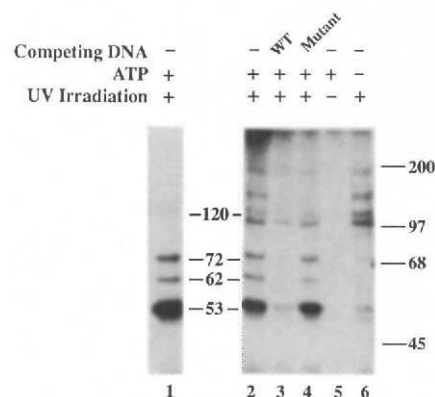


FIG. 5 Ultraviolet (UV) photocrosslinking of ORC polypeptides to *ARS1*. A DNA fragment of *ARS1*/848–855 (wild type for *ARS* function¹² and ORC DNA binding; Fig. 3) extending from position 734 to position 926 was modified with N_3 RdUMP at position 853 and with [32 P]dCMP at positions 852, 854 and 855 and used for ultraviolet photocrosslinking (4 ng per reaction). Lane 1 contains 16 ng glycerol-gradient-purified ORC; lanes 2–6 contain 80 ng partially purified ORC preparation following sequence-specific DNA affinity chromatography. All reactions contained 200 μ M ATP except 6. Reactions 3 and 4 contained 40 ng of an *ARS1* DNA fragment extending from position 852 to 926 and containing 35 base pairs of linker DNA 3' to the T-rich strand of the ACS. The fragment added to reaction 3 contained a wild-type (WT) ACS, whereas the fragment added to reaction 4 was derived from the ACS mutant *ARS1*/858–865 (ref. 12). All reactions were irradiated with UV light except 5. The positions of the M_r ($\times 10^{-3}$) size marker proteins are shown on the right. The migration of the corresponding unmodified ORC polypeptides is shown between the two panels.

METHODS. The substituted N_3 RdUMP probe was prepared as described²⁷ with the following changes: the *Hind*III to *Eco*RI fragment of p*ARS1*/WTA (ref. 12) was subcloned into *Hind*III- and *Eco*RI- cut M13mp19 replicative form DNA and the single-stranded DNA produced (the A-rich strand of the ACS) by the resulting phage was used as template. The annealed oligonucleotides used were the -40 universal sequencing primer and a second oligonucleotide hybridizing to the A-rich strand of the *ARS1* between positions 871 and 856. DNA binding as described in Fig. 2 legend, except the only nucleoside triphosphate added was ATP (200 μ M) and polyvinylalcohol was not added to the reactions. After binding, reactions were transferred to a microtitre plate and irradiated for 4 min with a short-wave UV light at a distance of 0.75 cm. Crosslinked proteins were treated with DNase (1 μ g) and micrococcal nuclease (7.5 U) and separated on a 10% SDS-polyacrylamide gel. Competitor DNA was prepared by cutting the *Hind*III-*Kpn*I fragment from the plasmids pABox/WT and pABox/858–865. These plasmids were prepared by cloning the *Bgl*II to *Kpn*I fragments of either p*ARS1*/WTA or p*ARS1*/858–865 (ref. 12) into pUC119 cut with the same enzymes.

been elusive. Our results make ORC a strong candidate initiator protein in yeast cells.

The DNA-binding specificity of ORC strongly supports this hypothesis. Point mutants within the ACS of *ARS1* that reduced ORC DNA binding in each case resulted in an equivalent reduction of *ARS1* function. At three other ARS elements the essential match to the ACS is also protected from DNase by ORC. Moreover, at all the ARS elements tested for ORC binding, the boundaries of the observed footprint correlate with the minimal sequences required for ARS function. Despite recognizing the ACS, ORC protects a much larger region of DNA extending predominantly 3' to the T-rich strand of the ACS. This asymmetric pattern of protection suggests that one or more of the ORC subunits are in an ideal position to act upon these essential 3' sequences. Although ORC does not specifically recognize the DNA 3' to the T-rich strand of the ACS, both DNA competition experiments (data not shown) and the alternating pattern of enhanced and protected DNase cleavage observed at these sequences suggest that important nonspecific contacts are made with this region of ARS elements. Consistent with this hypothesis, mutations that would be expected to disrupt the interaction of ORC with the 3' region, such as the precise inversion of the ACS, result in a loss of ARS function^{23,29}. Furthermore, the enhanced DNase cleavages observed at the 3' sequences suggests that the DNA is wrapped around ORC²² and is reminiscent of the pattern observed when the *E. coli* initiator protein *dnaA* binds to its cognate origin, *oriC*³⁰. Protein-DNA complexes involving wrapping have been implicated in the early events of DNA replication in several organisms^{31,32}.

The intriguing finding that ATP is required for specific DNA binding by ORC distinguishes it from most DNA-binding proteins. Of the sequence specific DNA-binding proteins known to bind ATP, many but not all (for example, see refs 33 and 34) are involved in the recognition of origins of replication, including SV40 and polyoma T-antigen, *dnaA* protein, bovine papillomavirus E1 protein, and herpes simplex virus UL9 protein (OBP)^{2,35}. Of these initiator proteins, only the DNA recognition properties of T-antigen are directly affected by ATP³⁶⁻³⁸. Studies of these initiator proteins suggest that ATP may have other roles in ORC function in addition to facilitating sequence-specific DNA binding. For example, ATP is required for the untwisting of the DNA helix by T-antigen and *dnaA* protein^{1,39}. ATP hydrolysis could also be important in regulation. Studies of *oriC* replication indicate that hydrolysis of ATP by *dnaA* protein results in the inactivation of its replicative properties^{40,41}. A similar mechanism involving eukaryotic initiator proteins could help prevent reinitiation during the S phase of the cell cycle. Further studies of the ATP requirement of ORC will be necessary to address these possibilities.

In addition to the results of the purification, the multiprotein nature of ORC is supported by the large size predicted by glycerol gradient sedimentation and the multiple proteins identified by ultraviolet photocrosslinking experiments (Fig. 5). Despite these findings, the exact composition of ORC remains to be confirmed. The development of new reagents, such as antibodies against the individual subunits, will help us understand the subunit structure of ORC. There is precedent for the use of multiple proteins to recognize an origin of DNA replication. The bovine papilloma virus initiator proteins E1 and E2 recognize their cognate origin of replication cooperatively³⁵. Multiprotein complexes are frequently assembled after origin recognition by an initiator protein². In yeast, ORC may obviate a subset of these assembly steps by maintaining a complex of proteins associated at all times.

The properties of ORC and the recently identified single-stranded DNA-binding protein(s) that recognize the ACS (refs 19-21) are distinct. While the single-stranded binding proteins recognize only the T-rich strand of the ACS, ORC recognizes the consensus sequence in its duplex form. In addition, the multiprotein nature of ORC, the ATP dependence of ORC DNA

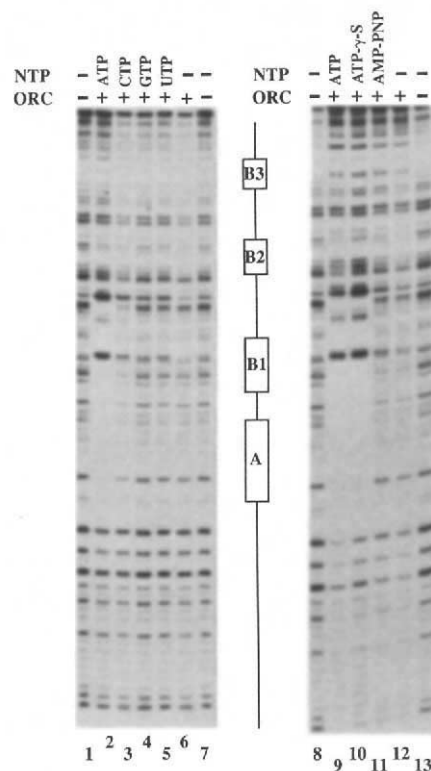


FIG. 6 ATP dependence of ORC DNA binding. *ARS1* DNA fragments labelled at position 926 and extending to position 734 were used for DNase footprinting (T-rich strand of the A element was labelled). Binding conditions were as described for Fig. 2 except that only a single indicated nucleoside triphosphate was added (at 80 μ M) and creatine phosphate and creatine phosphokinase were omitted from the reactions. The NTP added is shown above each lane. Reactions 2-6 and 9-12 contained 16 and 24 ng of ORC respectively. Reactions 1, 7, 8 and 13 contained no protein. The position of the elements of *ARS1* are indicated between the two panels.

binding, and the inability of ORC to alter the mobility of ACS-containing DNA fragments in non-denaturing polyacrylamide gels, all set it apart from the single-stranded DNA-binding protein(s). Although it remains possible that these single-stranded DNA-binding proteins function at *S. cerevisiae* origins of replication, on the basis of studies of bidirectional DNA replication in other organisms², it is unlikely that they function in the initial recognition of the origin. Instead, we propose that ORC recognizes the origin in its double-stranded form and that if the single-stranded DNA-binding proteins play any part, they are involved in events that occur after the DNA duplex is opened. Recent high-resolution *in vivo* footprinting experiments support the hypothesis that ORC mediates ACS function⁴⁶. These studies show a pattern of DNase protection at *ARS1* *in vivo* that is remarkably similar to the pattern induced by ORC *in vitro*, including the characteristically enhanced cleavages at B1 and B2.

If ORC is the *S. cerevisiae* initiator protein, its characterization will allow significant insights into eukaryotic DNA replication and its regulation. In addition to the information gained from understanding the properties of ORC itself, the availability of a eukaryotic initiator protein complex would provide a powerful tool for the development of a *in vitro* replication system directed by a chromosomal origin of DNA replication. ORC also would represent a prime target for proteins that regulate the initiation of S phase. Finally, if ORC does represent the yeast initiator protein, it is likely that related proteins will function at the origins of replication in metazoa. The identification and characterization of these related initiator protein complexes could, in turn, facilitate identification of chromosomal replicator

sequences in multicellular organisms, which remain poorly understood⁴². □

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Glutamate-induced long-term potentiation of the frequency of miniature synaptic currents in cultured hippocampal neurons

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Glutamate application at synapses between hippocampal neurons in culture produces long-term potentiation of the frequency of spontaneous miniature synaptic currents, together with long-term potentiation of evoked synaptic currents. The mini frequency potentiation is initiated postsynaptically and requires activity of NMDA receptors. Although the frequency of unitary quantal responses increases strongly, their amplitude remains little changed with potentiation. Tests of postsynaptic responsiveness rule out recruitment of latent glutamate receptor clusters. Thus, postsynaptic induction can lead to enhancement of presynaptic transmitter release. The sustained potentiation of mini frequency is expressed even in the absence of Ca²⁺ entry into presynaptic terminals.

LONG-TERM potentiation (LTP), an activity-dependent change in synaptic efficacy, may be essential to learning and memory in the mammalian brain^{1–5}. At hippocampal CA3–CA1 synapses, LTP is initiated by postsynaptic events that are relatively well understood^{5–14}. By contrast, the mechanism of expression of increased synaptic strength is controversial^{15–26}. Is presynaptic transmitter release enhanced during LTP? This possibility has been intensely debated since its first proposal by Bliss and colleagues¹⁵. A presynaptic component of LTP expression would require retrograde signalling from postsynaptic spines to presynaptic terminals^{2–5,15–19}, with important functional and developmental implications²⁷. In studies of evoked synaptic events, some laboratories reported large increases in quantal content, consistent with presynaptic enhancement^{16–19}, whereas another group found only increased quantal size²⁰. Rigorous quantal analysis is more difficult for excitatory transmission in the hippocampus^{15–26} than in other systems^{21,26,28–30}.

Miniature synaptic events (minis) are synaptic responses to

single packets of transmitter^{28–30}. Minis are generally seen as isolated events, aiding distinctions between quantal size and release probability, and have thus provided insights into synaptic plasticity^{31–38}. Changes in mini frequency and evoked responses occur in parallel during post-tetanic potentiation³¹ and perturbations of presynaptic signalling^{34–38}. Minis can be easily recorded in hippocampal neurons^{37,39–41} but their usefulness in understanding mechanisms of LTP has been hampered by uncertainty about whether minis arise from potentiated or non-potentiated synapses^{17,41}. Furthermore, findings of apparent changes in mini amplitude or frequency are by themselves ambiguous^{20,24,41} without direct tests of postsynaptic responsiveness. Increased quantal size might signify enhanced postsynaptic responsiveness or elevated presynaptic vesicular content; elevated quantal frequency might reflect enhanced vesicular release or recruitment of latent postsynaptic receptor clusters^{24,42}.

To overcome these problems, we used minis in combination with direct measurements of postsynaptic responsiveness. We obtained clear evidence that postsynaptic mechanisms of LTP induction can lead to sustained enhancement of presynaptic

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transmitter release. Our results also indicate the involvement of a mechanism for presynaptic enhancement apart from possible changes in Ca^{2+} entry.

LTP of evoked and spontaneous e.p.s.cs

Cultured CA3–CA1 hippocampal neurons form synapses with well developed spines (data not shown) and display NMDA receptor-dependent LTP¹⁷. Synapsin-I immunostaining revealed a few hundred terminals per postsynaptic cell (data not shown), $\sim 10^2$ -fold less than neurons *in situ*⁴³. Large CA1-like (spindle-shaped) pyramidal neurons were chosen for whole-cell recordings. Presynaptic neurons were focally stimulated with extracellular current or local application of K^+ -rich solution (Fig. 1 legend). To induce synaptic potentiation in a relatively homogenous population of synapses, a 50 μM glutamate, Mg^{2+} -free solution was locally applied for 30 s to the postsynaptic cell. This strongly increased evoked synaptic currents and raised the frequency of spontaneous events in 10/21 cells (Fig. 1a). In the 10 responding cells, evoked response amplitude rose 2.12 ± 0.4 -fold (mean $\pm$ s.e.m.) and spontaneous excitatory postsynaptic potential (e.p.s.c.) frequency increased 2.64 ± 0.55 -fold (20 min after induction relative to control). Both changes were maintained for the duration of the recording (up to 48 min after glutamate in Fig. 1b). Spontaneous e.p.s.c. frequency rose

promptly after glutamate application. Evoked synaptic currents often increased gradually (Fig. 1b), as in slices^{44,45}, probably because presynaptic glutamate receptors transiently inhibit presynaptic Ca^{2+} channels^{46–48} without affecting spontaneous release.

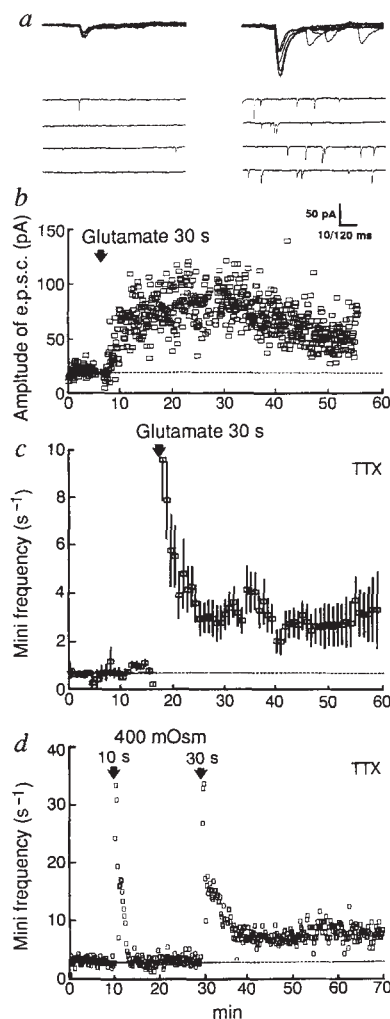
Mini frequency potentiation

All further experiments were done using 1 μM tetrodotoxin to block synaptic events arising from propagated action potentials. The minis that remained displayed a fast rise time (1–2 ms), decay time constants of ~ 5 ms, and could be blocked by CNQX. The NMDA component was easily observed only in the absence of external Mg^{2+} (ref. 40). Under these conditions, glutamate-induced potentiation of mini frequency was observed in 40/57 experiments; mini frequency increased 2.97 ± 0.32 -fold above control (5–10 min after glutamate application). In 26 cells that gave long-lived recordings, the average mini frequency rose to a sharp peak following removal of exogenous glutamate and subsided within 10 min to a steady rate, 3.9-fold greater than control (Fig. 1c).

Synaptic release of glutamate was also effective in potentiating mini frequency. Local application of hypertonic media was used to promote exocytosis^{40,49,50} (Fig. 1d). A 10-s hypertonic challenge with 400 mosM solution (Mg^{2+} -free) was followed by a

FIG. 1 Potentiation of evoked and spontaneous e.p.s.cs induced by a brief challenge with exogenous or synaptically released glutamate. *a*, Evoked responses (top) and spontaneous events (bottom). Left, control responses; right, responses 5 min after induction of potentiation by a 30-s challenge with 50 μM glutamate, Mg^{2+} -free tyrode. The evoked response amplitude rose from ~ 23 pA in control to ~ 70 pA; correspondingly, spontaneous e.p.s.c. frequency increased from 0.6 Hz to ~ 5 Hz. Both evoked and spontaneous e.p.s.cs could be blocked by 10 μM CNQX (6-cyano-7-nitroquinoxaline-2,3-dione). *b*, Amplitude of evoked responses plotted against time (same experiment as *a*). *c*, The time course of the mini frequency enhancement (1 μM tetrodotoxin (TTX) present). Ensemble average of mini frequency time-plots (bars are s.e.m., $n=26$). The number of experiments falls off to 2 at the end of the time period shown. Symbols plot mini frequency during 50-s epochs. *d*, LTP of mini frequency produced by synaptic release of glutamate. Vesicular release was elicited by 10-s and 30-s challenges with hypertonic (400 mosM) medium (Mg^{2+} -free tyrode solution supplemented with 100 mM sucrose). A 10-s hypertonic challenge gives only a short form of enhancement while the 30-s challenge produced LTP of mini frequency. 1 μM TTX, $V_m = -70$. Symbols plot mini frequency during 10-s epochs.

METHODS. Hippocampal regions CA3–CA1 were dissected out from 2–5-day-old rat brains, quickly subdivided into small pieces (~ 1 mm³), incubated for 4 min with trypsin type XI (10 mg ml⁻¹) plus DNase I type IV (0.5 mg ml⁻¹), and mechanically dissociated in Hank's solution supplemented with DNase type IV (0.5 mg ml⁻¹) and 12 mM MgSO_4 . The cells were recovered by centrifugation and plated onto polylysine-coated petri dishes (Corning, 30 mm) at a density of 25–50,000 per dish. Culture media contained: minimal essential medium, glucose 0.6%, glutamine 1 mM, NaHCO_3 2.4 g l⁻¹, bovine transferrin 100 μg ml⁻¹, insulin 25 μg ml⁻¹, fetal calf serum (FCS) 5–10%. Cultures were maintained at 37 °C in a 95% air, 5% CO_2 -humidified incubator. The culture media was replaced every 4 days. From the second day in culture, the media was supplemented with cytosine- β -D-arabinofuranoside (1–5 μM). The cultured neurons were not treated with glutamate receptor blockers. The incidence of functional autapses in this culture system was very low, judging by the absence of any change in mini frequency with steady depolarization. Whole-cell recordings were obtained at room temperature (23–25 °C) from pyramidal cells with spindle-shaped, CA1-like morphology (10–14 days after cell plating), with an EPC-7 patch clamp amplifier (LIST). The input resistance (~ 0.5 –2 G Ω) was monitored during the course of the experiment. The bath was perfused continuously with oxygenated control tyrode (2 ml min⁻¹). Test solutions were delivered to the postsynaptic cell with an array of capillary tubes (internal diameter 300–500 μm) positioned ~ 10 –50 μm from the cell. To induce mini frequency potentiation, the postsynaptic cell was exposed to Mg^{2+} -free tyrode for a few seconds, then glutamate (25–50 μM) in Mg^{2+} -free tyrode for 30 s, followed by control solution. Patch electrodes (5–10 M Ω resistance) contained (in mM): caesium gluconate 102, MgCl_2 5, NaCl 10, Cs-EGTA 0.6, ATP 2, GTP 0.2, HEPES 49 at pH 7.2. Control tyrode solution contains (in mM): NaCl 119, KCl 5, CaCl_2 2, MgCl_2 2, glucose 30, picrotoxin 0.1, glycine 1 μM , HEPES 25 at pH 7.4. External solution contained 1 μM TTX unless stated otherwise. For data



analysis, the tape-recorded current signal was digitized at 10 kHz and stored on a PDP-11/23 computer. Statistical comparisons were made with Student's *t*-test. All chemicals Sigma except tetrodotoxin and transferrin (Calbiochem) and MK801 (gift of D. W. Choi). Culture media were obtained from GIBCO and the FCS from Hi-Clone (Grand Island, New York).

decrementing (~ 3 min) increase in mini frequency. A 30-s hypertonic challenge produced a transient peak, followed by a sustained, threefold enhancement in mini frequency. Sucrose-induced potentiation was observed in 4/4 cells and lasted for the duration of the recording (up to 40 min after sucrose).

Postsynaptic induction

LTP in area CA1 is initiated by Ca^{2+} entry through postsynaptic NMDA-receptor channels²⁻⁷. Likewise, mini frequency potentiation required Ca^{2+} entry and potentiation was not seen if glutamate was applied in Ca^{2+} -free solution (100 μM EGTA, 4 mM Mg^{2+} ; $n=7$). Involvement of the postsynaptic cell was tested by varying its membrane potential (V_m) during glutamate application (0.5–1.0 mM Mg^{2+} present). Hyperpolarization to -110 mV during the glutamate application suppressed the mini frequency increase (assessed at -70 mV, Fig. 2a); subsequent reapplication of glutamate at -10 mV elicited potentiation (Fig. 2b). With hyperpolarization, mini frequency potentiation was not seen in 30 of 32 cells; clear potentiation was then observed in 7 of 15 cells when glutamate was reapplied with $V_m \sim 0$ mV (Fig. 2c).

The influence of postsynaptic membrane potential is consistent with voltage-dependent Mg^{2+} block of NMDA receptor channels⁵⁻⁷. Accordingly, we tested MK801, a selective blocker of the NMDA receptor pore. With MK801 present, application of glutamate failed to increase mini frequency (15 of 15 cells); a subsequent rise in mini frequency was obtained in six of eight experiments in which glutamate was successfully reapplied (Fig. 2c).

Quantal properties

Figure 3 compares mini e.p.s.c. amplitude during a 13 min control period (a) and 10–23 min after induction of mini frequency potentiation (b). Both amplitude distributions show a broad dispersion and skewed shape⁴⁰. The number of events increased twofold with potentiation, but there was no change in mini time course (Fig. 3a, lower inset) or mean mini amplitude (4.1 pA in both a and b); the shape of the amplitude distribution also remained unchanged (note normalized control histogram in b). Figure 3b (inset) illustrates data from 19 experiments with

enough control events (>500) to obtain a reliable amplitude histogram. Only two cells showed an appreciable rise in mean mini amplitude after mini frequency potentiation. On average, there was no significant difference in mean mini amplitude (Fig. 3b, inset; $P > 0.4$). Collected cumulative amplitude distributions showed no significant change with potentiation at any percentile (Fig. 3c). By contrast, increasing the driving force for inward synaptic currents by postsynaptic hyperpolarization produced a significant and uniform increase in mini amplitude (7/7 experiments, Fig. 3d), demonstrating that changes in amplitude were readily detectable.

Hyperpolarization never increased the apparent mini frequency, indicating that the mini frequency potentiation cannot be attributed to the enlargement of minis previously sub-threshold for detection. This point is reinforced by the finding that potentiation left the mini time course unchanged (Fig. 1a, lower inset).

Tests of postsynaptic responsiveness

Application of exogenous ligands provided another important test for changes in postsynaptic responsiveness. Glutamate or 2-(aminomethyl)phenylacetic acid (AMPA) (2 mM Mg^{2+}) were applied through a local puffer pipette to a postsynaptic area in the region where the enhancement was induced (Fig. 4). The postsynaptic currents showed no change (Fig. 4a, b), in contrast to the clear mini frequency potentiation (Fig. 4c). This was a consistent finding in 6/6 cells (up to 38 min after potentiation). The mini frequency increased 1.95 ± 0.25 -fold ($P < 0.02$, 15–20 min after induction) whereas the ratio of puffer response amplitudes was unchanged (1.01 ± 0.07 , $P > 0.5$; 4 cells tested with glutamate, 2 cells with AMPA). Our measurements of glutamate or AMPA responsiveness were limited to the first 30–40 min after inducing LTP of mini frequency. It remains to be seen whether a delayed increase in AMPA responsiveness emerges at later times, as found in hippocampal slices⁵¹.

These results are also relevant to the hypothesis that LTP results from all-or-nothing recruitment of inactive postsynaptic receptor clusters²⁴ (Fig. 5a). The experiments in Fig. 4 directly assess responsiveness to exogenous ligands but may include some contribution from extrasynaptic receptors. To focus more

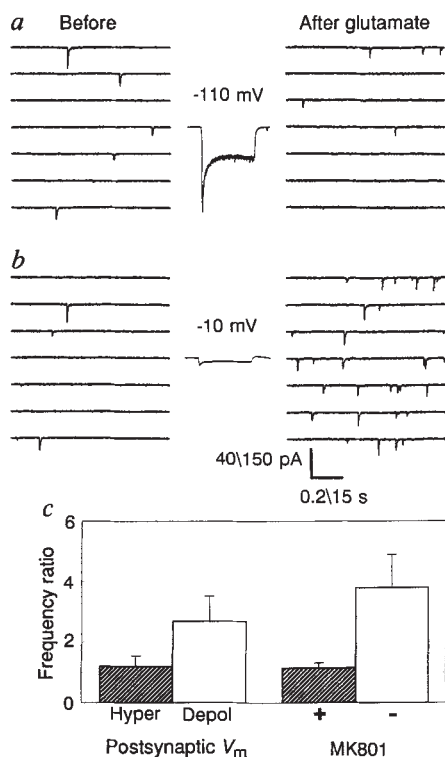


FIG. 2 Potentiation of mini frequency is prevented by postsynaptic hyperpolarization or NMDA receptor blockade. a, Representative consecutive current traces ($V_m = -70$) obtained before (left) and 5 min after (right) a 30-s glutamate pulse (50 μM glutamate in 0.5 mM Mg^{2+} tyrode). $V_m = -110$ mV during the glutamate pulse. No increase in mini frequency. b, Same cell, $V_m = -10$ mV during the glutamate pulse. Note the pronounced potentiation of mini frequency. Shown in the centre of a and b are inward currents elicited at -110 and -10 mV by the glutamate pulses (note lower current gain, slower time base). c, Collected data on effects of membrane potential (left), and the NMDA-receptor blocker MK801 (right). Bars plot mean values ($\pm$ s.d.) for the ratio of mini frequency (10 min after glutamate challenge/control). Left, mini frequency ratio was 1.2 ± 0.34 when glutamate was applied at a strongly negative holding potential ($-100/-120$, shaded bar) and 2.69 ± 0.83 when glutamate was applied with $V_m \sim 0$ mV (open bar); $P < 0.05$, $n=7$. Right, ratio of mini frequencies was 1.15 ± 0.17 when glutamate was applied in the presence of 10 μM MK801 (shaded bar), and 3.8 ± 1.1 when the induction procedure was repeated after washing out MK801 (open bar); $P < 0.05$, $n=8$. MK801 ((+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate) is a non-competitive blocker of NMDA receptor channels and would not be displaced by the high concentrations of glutamate used in the induction procedure, unlike competitive receptor blockers such as APV (D-2-amino-phosphonovalerate).

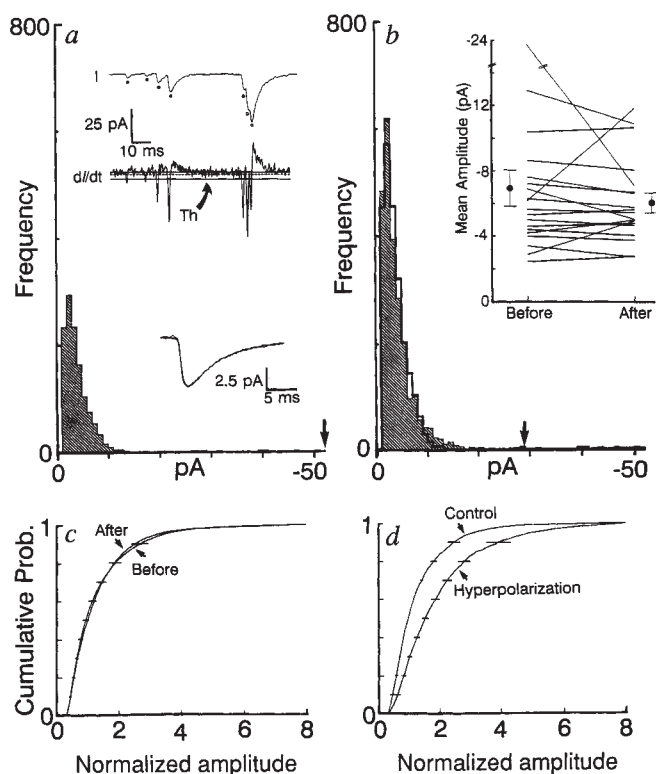


FIG. 3 Effects of glutamate-induced potentiation and membrane potential on the amplitude distribution of minis. *a*, Histogram of peak mini amplitude during a 13-min control period ($n=1,230$). Mean amplitude, 4.1 pA. Upper inset, records of current and its first derivative (dI/dt) to illustrate the automated detection of minis. Minis were detected by a combination of two criteria: first, the first derivative must exceed a threshold (Th), ~ 3 times the s.d. of the background dI/dt (dashed line, determined from blank sweeps), and second, the peak amplitude of the mini must exceed a preset threshold (usually ~ 1 –2 pA). Events arising from the summation of individual minis (arrow) were excluded from estimates of peak amplitude by requiring a minimal interval of time between a clear peak of the first event and the rise of a clear second event. Solid circles mark detected minis (for example, first two events have amplitudes of ~ 2 pA). Open circles indicate events of undetermined amplitude that were included in estimates of mini frequency. Note that this procedure minimizes the chances that near-simultaneous events will be erroneously lumped together as a large mini and also allows the detection of a small mini on the falling phase of a large one. Lower inset, superimposition of normalized averages of 40 minis taken before and after potentiation, showing no change in mini time course. *b*, Histogram (shaded) from the same experiment as *a*, 10–23 min after induction of LTP with a glutamate puff. Mini frequency doubles ($n=2,396$) but the overall shape of the distribution is maintained (mean amplitude, 4.1 pA). Solid line shows the control histogram normalized to same area. $V_m = -70$ mV. Vertical arrows in *a* and *b* mark the largest mini detected during the respective recording periods (-52 pA in *a*, -29 pA in *b*). Inset in *b*, mean amplitude before and 10 min after the induction of LTP of mini frequency in 19 individual experiments. The filled circles represent the group means $\pm$ s.e.m.: 6.94 ± 1.1 pA before potentiation, 6.1 ± 0.6 pA 10 min after glutamate application. The s.d. of the background noise in these experiments was ~ 0.7 – 1.2 pA. *c*, Averages of cumulative amplitude distributions (19 cells, as in *b*, inset) obtained before and 10 min after induction of mini frequency potentiation. The distributions were normalized by the median control value for each experiment. The normalized amplitudes were then averaged at each percentile and plotted horizontally. Error bars indicate mean $\pm$ s.e.m. Notice the almost perfect overlap of the two curves. *d*, Averages of cumulative amplitude distributions showing effect of a 30 mV hyperpolarization (7 cells). Method of analysis similar to *c*. The curve on the left shows the average distribution at -60 mV/ -70 mV; curve on the right, data at -90 mV/ -100 mV. The average distribution is shifted to the right over the entire range of amplitudes by the 30 mV hyperpolarization. These experiments were done in control tyrode supplemented with (mM) CsCl 1, MnCl₂ 2, BaCl₂ 1, and tetraethylammonium chloride (TEA-Cl) 10, to aid long-lasting lower noise recordings at negative potentials. This made it possible to collect enough events for a reliable histogram (the average number of events per distribution was $\sim 1,000$).

specifically on synaptic receptors, we used a complementary approach (Fig. 5*b, c*). Vesicular release of glutamate from presynaptic nerve terminals was provoked by brief hypertonic challenges (sucrose, 2 mM Mg²⁺) and mini frequency was measured. If potentiation arises from recruitment of latent receptor clusters, the response to enhanced vesicular release should be potentiated to the same degree as basal mini frequency. This was not the case (Fig. 5). Whereas basal mini frequency increased 22-fold above control (5–10 min after induction), mini frequency during hypertonic challenges increased only 1.1-fold. Similar results were obtained in 6 of 6 experiments: spontaneous rates rose 8.46 ± 1.9 -fold, whereas mini frequency in hypertonic solution increased only 1.15 ± 0.34 -fold. The maximal mini frequency was well below limits of detection. Estimates of mini frequency were supported by measurements of total charge transfer during sucrose challenges (Fig. 5*b*). These results confirm that the mini frequency potentiation originates presynaptically and provide new information about the underlying mechanism. The observation that the response to hypertonic challenges fails to scale with the glutamate-induced potentiation (Fig. 5*c*) indicates that both interventions produce changes along a final common pathway leading up to vesicular release.

Sustained elevation of Ca²⁺ entry?

As a further exploration of possible presynaptic mechanisms, Fig. 6 illustrates the effect of external Ca²⁺ removal on mini frequency before and after the induction of potentiation. After glutamate application, mini frequency was elevated eightfold. The potentiation was not reduced by applying Ca²⁺-free 4 mM Mg²⁺ solution (4 of 4 experiments). Thus, mini frequency

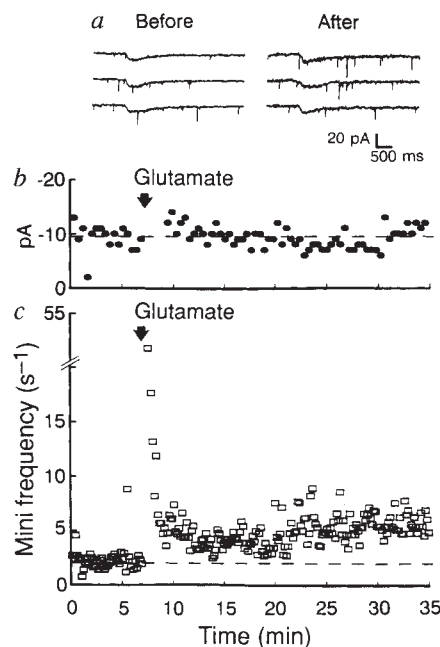


FIG. 4 Potentiation of mini frequency is not accompanied by enhanced postsynaptic responsiveness to exogenously applied glutamate. *a*, Representative consecutive current traces obtained with brief puffer applications of 50 μ M glutamate (2 mM Mg²⁺) before (left) and 10 min after (right) the induction of mini frequency potentiation. The wave of inward current is the response to a 10 ms puff of glutamate solution (and was completely abolished by 10 μ M CNQX). Individual minis appear as brief downward spikes on the compressed timescale. Note the marked increase in the number of minis after LTP induction without any significant increase in the amplitude of the responses to glutamate puffs. Similar results were obtained with puffer application of 50 μ M AMPA. *b*, Amplitude of postsynaptic responses to glutamate application plotted against time. Arrow marks 30-s pulse of 50 μ M glutamate (Mg²⁺-free). *c*, Timecourse of the mini frequency potentiation, measured over 10-s epochs, from the same experiment.

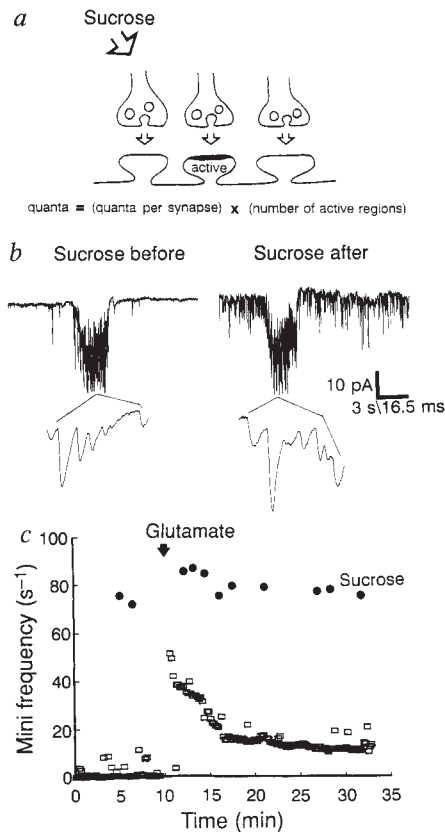


FIG. 5 Stimulation of synaptic transmitter release by hypertonic solution and mini frequency potentiation are not independent. **a**, Schematic illustration of the hypothesis that LTP arises from recruitment of inactive synapses or latent receptor clusters^{24,41}. Mini frequency can be expressed as a product, $F_{tot} = f_{pre} \times AR$ where f_{pre} = rate of vesicle release per synaptic region and AR = the number of active postsynaptic regions; f_{pre} is increased by hypertonic challenges that provoke vesicular release. If glutamate-induced potentiation were due to an independent increase in AR , this increase should be multiplicative with changes in f_{pre} . **b**, Responses evoked by 3-s hypertonic challenges (2 mM $MgCl_2$ tyrode plus sucrose, final osmolarity 500 mOsm), before and ~3 min after the induction of LTP of mini frequency. Hypertonic challenges were applied with the same solution delivery system used to induce the potentiation. Increased transmitter release during hypertonic challenge is reflected by enhancement of current fluctuations (expanded traces below) and increased inward current. $V_m = -70$. **c**, Time course of the background spontaneous mini frequency (■) as well as the frequency response to the sucrose challenges (●) before and after the induction of potentiation (arrow). The basal mini frequency rose from the control value of 0.74 Hz to 16.4 Hz between 5 and 10 min following induction. By contrast, mini frequency evoked by hypertonic challenges barely increased, from a mean of 72 Hz to 78 Hz. Tests were performed to show that mini frequencies could be faithfully detected with little or no saturation. Measurements of the total charge transfer during the sucrose application (**b**) are in agreement with this interpretation. Mutual occlusion between effects of hypertonicity and glutamate-induced mini frequency potentiation points to effects along the same final common pathway.

potentiation cannot be explained by a sustained elevation of Ca^{2+} entry, but would be consistent with an alteration of the internal machinery controlling transmitter release.

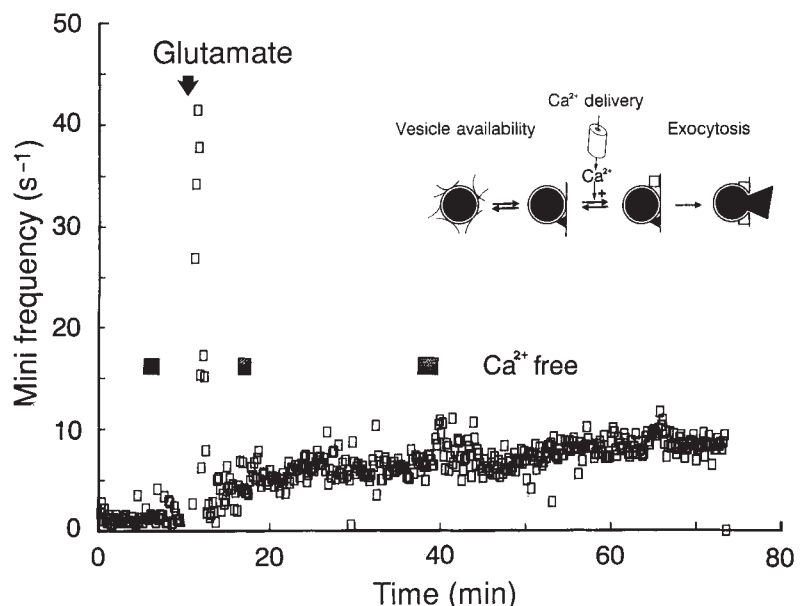
Discussion

Mini frequency potentiation is associated with sustained enhancement of evoked transmission and is induced by mechanisms similar to those underlying LTP of evoked transmission in area CA1 (refs 1–10). Thus, mini frequency potentiation is: (1) large and enduring; (2) can be induced by provoking endogenous neurotransmitter release; (3) requires Ca^{2+} entry

for induction; (4) is prevented by blockade of NMDA receptor channels; and (5) can be blocked by strong postsynaptic hyperpolarization.

Although the induction of the potentiation is postsynaptic, the locus of expression is clearly presynaptic under the conditions of our experiments. During the large and sustained increase in mini frequency, mini amplitude showed no consistent change in most experiments. Even small changes in amplitude would have been easy to detect under the conditions of our experiments (Fig. 3 legend). These data are reinforced by additional tests of postsynaptic responsiveness. Currents evoked by direct applica-

FIG. 6 Effect of Ca^{2+} removal on LTP of mini frequency. Mini frequency time-plot obtained by averaging mini frequency in 10-s epochs, before and after the glutamate application (arrow, 50 μM glutamate, Mg^{2+} -free for 30 s). Superfusion of the cell with Ca^{2+} -free 4 mM Mg^{2+} solution for up to 3 min (solid bars) by means of the same fast-flow system used to deliver glutamate did not diminish mini frequency before or after potentiation. The Ca^{2+} -free 4 mM Mg^{2+} solution was made with distilled deionized water (Nanopure) with no added EGTA (long exposure to EGTA increased nonspecific leak current and caused cell damage). The efficiency of the Ca^{2+} removal procedure was confirmed by the abolition of evoked responses (tested in the absence of TTX, data not shown). There was a small transient increase in mini frequency following restoration of 2 mM Ca^{2+} /2 mM Mg^{2+} external solution (off response) that was more pronounced in other experiments. The off response is probably an index of a transient rise in intraterminal internal Ca^{2+} concentration following restoration of external Ca^{2+} , and suggests that mini frequency is sensitive to increases in internal Ca^{2+} concentration. Inset, general scheme of the main steps involved in the exocytotic process, possible targets of mechanisms for expression of LTP.



tion of exogenous glutamate or AMPA remained constant; the response to synaptically released glutamate (provoked by hyper-tonic challenges) was also little changed. All three lines of evidence point toward a sustained enhancement of quantal transmitter release with no appreciable change in postsynaptic responsiveness.

In only rare cases (2 of 19 cells; Fig. 3) did we find a significant rise in mini amplitude, comparable to the 15–60% enhancement of mini amplitude reported by Manabe *et al.*⁴¹. In both of these cells, the mini frequency increase was ~300%. It is possible that increases in release probability or quantal amplitude correspond to distinct mechanisms expressed to varying degrees under different experimental conditions. The largest increases in quantal amplitude reported^{20,41} fall short of the enhancement that can be seen during LTP^{16,17,19}. By contrast, changes in quantal content (or mini frequency) can be quite large, and are likely to be the dominant mechanism when the potentiation is several-fold^{16–19}.

Our results provide a stringent test of the hypothesis that increases in quantal content arise from recruitment of inactive postsynaptic receptor clusters^{24,26}. This was addressed by repeatedly provoking bursts of neurotransmitter release, and counting quanta to look for changes in the number of functional postsynaptic units (Fig. 5). During marked potentiation of basal mini frequency, the number of quanta per presynaptic challenge barely increased. This argues strongly against appreciable activation of latent receptor clusters.

Enhanced spontaneous glutamate release may also have relevance to excitotoxicity. Our recordings consistently showed a large, early increase in mini frequency, decaying within ~10 min after removal of exogenous ligand. Similar enhancement of spontaneous transmitter release onto neurons not under voltage clamp would promote continued postsynaptic Ca^{2+} entry, possibly leading to cell death⁵². This might explain why excitotoxicity in cultured neurons can be reduced by NMDA antagonists over a critical period <15 min after removal of exogenous ligand⁵³.

These results provide evidence that postsynaptic induction mechanisms can lead to enhancement of presynaptic function. This experimental system offers advantages for testing putative retrograde messengers such as nitric oxide^{27,54,55}. Indeed, brief application of nitric oxide produces a sustained increase in mini frequency in hippocampal cultures⁵⁵. Furthermore, our experiments give clues about possible targets in the presynaptic terminal beyond the retrograde signal. The near occlusion between glutamate-induced potentiation and presynaptic manoeuvres (Fig. 5) suggests actions along a final common pathway leading to transmitter release. Furthermore, the analysis of minis helps distinguish between possible modifications of Ca^{2+} delivery or Ca^{2+} responsiveness of the release machinery³³. Although a modification of presynaptic Ca^{2+} delivery is not excluded, our experiments point to the existence of another neurosecretory modification that enhances synaptic vesicle availability or efficiency of exocytotic fusion (compare with refs 33–38). □

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Necessity of evolution in cosmological γ -ray bursts

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RESULTS from the Burst and Transient Source Experiment (BATSE)¹ on the Compton Gamma-Ray Observatory show a relative deficiency of faint γ -ray bursts without any detectable anisotropy. This gives strong support to suggestions that the bursts originate at cosmological distances², such that cosmic expansion reduces the detectability of faint (and therefore distant) sources relative to a uniformly filled euclidean space. But the Pioneer Venus Orbiter (PVO) observations of brighter bursts show no such deficiency, indicating that the bursts that it sees are at redshifts less than about one. The best-fit cosmological model based on PVO data (peak luminosity $L_0 = 2 \times 10^{50}$ erg s⁻¹) with no evolution of the source population predicts, when extrapolated to the fainter sources, a factor of 40 more events than BATSE sees. Even the 3σ limit from PVO ($L_0 = 9.1 \times 10^{51}$ erg s⁻¹) is barely consistent with the BATSE result. Evolution of the cosmological γ -ray burst sources with epoch therefore seems necessary if the PVO and BATSE data are to be reconciled. The required evolutionary trend is for fewer or fainter bursts at larger redshift.

There have been suggestions^{3,4} that γ -ray bursts are caused by cosmic strings originating at redshifts as large as 1,000. Alternatively, colliding compact objects with modest redshifts (~ 1) have been assumed, implying luminosities of $\sim 10^{51}$ erg s⁻¹ (ref. 2). The brightness distribution of γ -ray bursts observed by the PVO can put stringent limits on these models. The number of bursts N observed above a peak flux P by the PVO experiment (the log N -log P distribution) follows a $-3/2$ power law for a range of more than a factor of 30 in intensity⁵⁻⁸. This distribution is that expected for a homogeneous source distribution in euclidean space. In contrast, the BATSE experiment has in general observed much weaker bursts, and its log N -log P distribution is much flatter than a $-3/2$ power law¹. Some log N -log P investigations of γ -ray bursts have produced ambiguous results because they depended on determining N near the threshold of the detector where instrumental effects are overwhelming. In contrast, here we use only the portion of the log N -log P curve for which threshold effects are unimportant, to place an upper limit on the presence of cosmological effects.

Between 1978 and 1991, PVO observed 273 triggered events with peak fluxes above 10^{-5} erg cm⁻² s⁻¹ in a bandpass of 100 keV to 2 MeV. The events include those between 1978 and 1988, which have been studied previously⁵⁻⁸. A PVO burst with a peak flux of 10^{-5} erg cm⁻² s⁻¹ typically has a fluence of 2.8×10^{-5} erg cm⁻². Threshold effects are significant for fluxes less than $\sim 2 \times 10^{-5}$ erg cm⁻² s⁻¹. The best-fit power law for fluxes $> 2.2 \times 10^{-5}$ erg cm⁻² s⁻¹ has a slope of -1.52 ± 0.18 , consistent with no cosmological effects⁷. Here, the confidence region was found by the methods described in ref. 9, although such methods assume normal distributions rather than Poisson distributions. We have used Monte Carlo techniques to verify that, in this case, the differences between Poisson and normal distributions have only a small effect⁷. To find the extent to which cosmological models can fit the PVO log N -log P distribution, we divided it into eight logarithmically spaced intervals from 2.2×10^{-5} to 7.0×10^{-4} erg cm⁻² s⁻¹. This eliminates the 75 weakest and the two brightest events, leaving 197 events. Intervals have 16-31 events.

The method of predicting the number of events in each interval resembles calculations for other cosmological sources (such as quasars), except that PVO detects photons over a limited bandpass so the simple bolometric distance modulus is inappropriate.

If we let $\phi(E)$ be the spectrum of the peak emission in the comoving frame (in photon s⁻¹ keV⁻¹), then the peak intensity observed by the PVO experiment is

$$P = \frac{\int_0^\infty \varepsilon(E) E \phi[(1+z)E] dE}{4\pi r_z^2} \quad (1)$$

where $\varepsilon(E)$ is the PVO efficiency and the comoving radial distance, r_z , is¹⁰

$$r_z = \left(\frac{c}{(1+z)q_0 H} \right) (zq_0 + (q_0 - 1)(-1 + (2q_0 z + 1)^{1/2})) \quad (2)$$

where H is the Hubble constant and q_0 is the deceleration parameter. We will take $H = 75$ km s⁻¹ Mpc⁻¹ and $q_0 = 1/2$. In the limit that $\varepsilon(E)$ is unity for all energies, the numerator of equation 1 is $(1+z)^{-2}$ and one obtained the relationship for bolometric observations. If $\phi(E)$ has the form $E^{-\alpha}$ and if $\varepsilon(E)$ is unity in the PVO bandpass and zero otherwise, the intensity at PVO is roughly

$$P = 1.3 \times 10^{-58} (1+z)^{-\alpha+2} L_0 / (1+z - (1+z)^{1/2})^2 \quad (3)$$

where L_0 is the standard-candle peak luminosity (in erg s⁻¹) in the PVO bandpass of E_1 to E_2 (100 to 2,000 keV). That is,

$$L_0 = \int_{E_1}^{E_2} E \phi(E) dE \quad (4)$$

For each PVO interval bounded by P_i and P_{i+1} , we solved equation 3 for the corresponding z_i and z_{i+1} . Then, equation 2 gives r_{z_i} and $r_{z_{i+1}}$ such that the number of predicted events in an interval is

$$\begin{aligned} \Delta N_i &= \int_{r_{z_i}}^{r_{z_{i+1}}} (4\pi\rho/(1+z)) r_z^2 dr_z \\ &= \int_{r_{z_i}}^{r_{z_{i+1}}} (\pi\rho(r_z - 2R_0)^2/R_0^2) r_z^2 dr_z \end{aligned} \quad (5)$$

where $R_0 = c/H$. For now, we have assumed that the number of bursts per comoving volume (ρ) is a constant (not evolving with epoch) and that L_0 is the same for all bursts (a δ -function luminosity distribution). The $(1+z)^{-1}$ factor is necessary¹¹ to account for the reduction in the burst frequency at earlier epochs. We assumed a typical value of 2 for α and varied the two free parameters (L_0 and ρ) to find the best-fit model. The best-fit

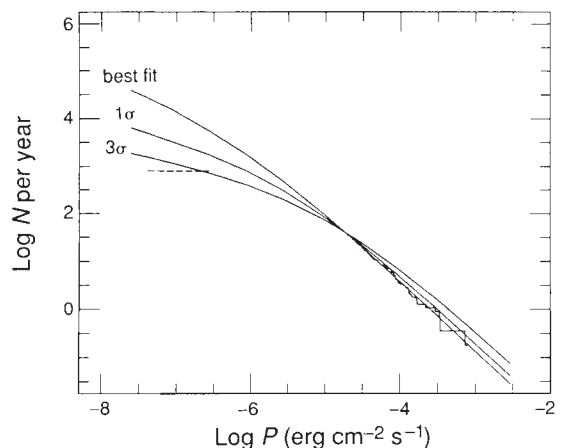


FIG. 1 Cosmological models fitted to results from PVO. The histogram is the log N -log P distribution for 1978 to 1991 above 2×10^{-5} erg cm⁻² s⁻¹. The three curves represent the best fit, the 1σ fit and the 3σ fit to the PVO data. The dashed line is the range of possible positions for the BATSE limit⁴. The inconsistency between the extrapolation of the PVO curves and the BATSE result indicates evolution with epoch of the γ -ray burst progenitor.

value of L_0 is $2 \times 10^{50} \text{ erg s}^{-1}$ with a chi-squared value of 2.39 for 6 degrees of freedom (probability = 12%). The best-fit event density is $\sim 3.1 \times 10^4$ events per year within a Hubble distance, that is, within a volume of $4\pi R_0^3/3$.

In Fig. 1, we show as a histogram the PVO log N -log P distribution for fluxes $> 2 \times 10^{-5} \text{ erg cm}^{-2} \text{ s}^{-1}$. The curve labelled 'best fit' was generated by equations 2, 3 and 5 and extends the model to the parameter range of the BATSE experiment. The relative sensitivities of the two experiments are uncertain. BATSE's event rate is quoted as ~ 800 events per year above a threshold of $10^{-7} \text{ erg cm}^{-2} \text{ s}^{-1}$, presumably in the 50–300 keV bandpass¹. For a typical γ -ray burst spectrum, this threshold would be equivalent to $\sim 5 \times 10^{-8} \text{ erg cm}^{-2} \text{ s}^{-1}$ in the PVO bandpass. An alternative normalization considers that the PVO event rate is achieved by BATSE at a factor of ~ 40 above its threshold¹. This implies that the BATSE threshold is at $\sim 3 \times 10^{-7} \text{ erg cm}^{-2} \text{ s}^{-1}$ in the PVO bandpass, a value which seems rather high. The dashed line in Fig. 1 shows the range of the possible positions for the BATSE limit given the uncertainty in the relative normalization. Future work should be able to resolve this uncertainty. Our current normalization of the PVO log N -log P distribution is 38 events per year per 4π above $2 \times 10^{-5} \text{ erg cm}^{-2} \text{ s}^{-1}$ in the 100–2,000 keV bandpass. Note that the BATSE limit is a factor of 30 – 40 to ~ 40 below what would be expected from the PVO best fit. Also shown in Fig. 1 are the 1σ and 3σ fits to the PVO log N -log P distribution. The 1σ fit is at $1.8 \times 10^{51} \text{ erg s}^{-1}$ with 1,770 events per year within a Hubble distance. The 3σ fit is $L_0 = 9.1 \times 10^{51} \text{ erg s}^{-1}$ with 300 events per year. Even the 3σ curve is barely consistent with the BATSE limit.

Given the range over which PVO has a slope near $-3/2$, it is difficult to find cosmological models without evolution that bend over fast enough to accommodate the BATSE observations. Source evolution with epoch (that is, $\rho(z)$ varies with z) is an explanation that allows PVO and BATSE to be consistent. It is not unrealistic to expect evolution, especially for models¹² that involve the collision of two compact objects. Note that for $L_0 = 10^{51} \text{ erg s}^{-1}$, events at $2 \times 10^{-5} \text{ erg cm}^{-2} \text{ s}^{-1}$ in PVO would be at $z = 0.15$. The right and left ends of the dashed line in Fig. 1 would be at $z = 1.1$ and $z = 2.6$, respectively. These values bracket the estimate of ref. 11 (1.7) for value of z at the BATSE threshold. Evolution could be completely dominating the brightness distribution, allowing L_0 to be smaller than $10^{51} \text{ erg s}^{-1}$ and allowing most events to be at small z . If γ -ray bursts were due to cosmic strings at large redshifts^{3,4} then the peak luminosities would be much larger and the shape of the distribution for the brightest events would be similar to that for the 3σ curve on the left in Fig. 1, that is, rather flat. The $-3/2$ power law for the (presumably) closest events argues that they are in euclidean space and not at extreme redshifts.

We have investigated two key assumptions to see if they could reconcile the results without evolution. First, we consider the spectral shape. In deriving the curves of Fig. 1, we took the spectral shape to be a power law with $\alpha = 2$, a typical value observed above a few hundred kiloelectronvolts. Using $\alpha > 2$ aggravates the discrepancy between PVO and BATSE. Using $\alpha < 2$ implies that the spectrum must roll over at high energies to a slope with $\alpha > 2$ to avoid having the integrated power diverge. Nevertheless, we varied the spectral shape from $\alpha = 2$ to $\alpha = 1$. Although the 3σ fit for L_0 increased by a factor of 2, it only moved the extrapolated PVO curve 15% closer to the BATSE limit. Second, we included a luminosity function by using Monte Carlo techniques, as in our preliminary report⁷. A source was assumed to be capable of producing peak rates that varied uniformly by a factor of 10. Power-law luminosity functions would be expected to give results closer to the original δ -function assumption. The 3σ fit for L_0 increased by a factor of 1.6, less than that due to varying α . Thus, neither spectral shape nor a broad luminosity function seems to be capable of reconciling the discrepancy.

It has been claimed^{11,12} that the BATSE and PVO results are consistent without source evolution. Those analyses used only the total event rates for PVO and BATSE without confidence regions, and as there are two free parameters (L_0 and ρ), it might always be possible to find an acceptable fit. Indeed, $L_0 = 2.7 \times 10^{52}$ and $\rho = 122$ events per year within a Hubble distance produce 800 and 38 events per year in BATSE and PVO, respectively, but the resulting shape of the distribution in PVO is unacceptable. Explanations of the BATSE brightness distribution that assume no evolution are probably invalid.

Finally, our limit on L_0 is an upper limit on the luminosity (assuming no evolution). The lack of observable galaxies¹³ in γ -ray burst error boxes has been used to put a lower limit on L_0 of $10^{52} \text{ erg s}^{-1}$ (but only galaxies like M31 were included). As our 3σ upper limit is equal to this previous lower limit, taking these two results at face value leaves little or no acceptable range for the luminosity of γ -ray bursts if they are at cosmological distances. $\square$

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Loss of homogeneity in a suspension by kinematic action

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SEPARATION of heavy particles from a fluid medium by centrifugal forces or by barodiffusion is well known¹. For a homogeneous fluid mixture comprising a suspension of very fine particles (such as smoke in air), on the other hand, for which the centrifugal and barodiffusive effects are negligible, a continuum model of the suspension would suggest that it is not possible to separate the particles from the fluid by flow-induced kinematic action alone. Here we present experimental evidence to the contrary: we have observed spontaneous segregation of smoke particles from air in an initially uniform mixture, when rotational flow is induced in the medium. A definitive explanation for this effect is not yet forthcoming.

For our experiments we used a vortex breakdown chamber similar to that used by Escudier² (Fig. 1a), in the hope that the converging and diverging streamlines in the vortex breakdown region would separate a homogeneous mixture by kinematic action. The height H of the chamber was 13.97 cm and its radius R was 7.63 cm, so that the aspect ratio H/R was 1.83. We used a homogeneous mixture of air and tobacco smoke and worked at a Reynolds number $Re (= \Omega R^2/\nu) = 2,500$ (where Ω is the angular speed of the bottom plate and ν is the kinematic viscosity). These values of H/R and Re fall within the range of values that produce a bubble by vortex breakdown². After smoke injection the flow chamber was hermetically sealed so that fluid

could neither enter nor leave the domain. The evolution of the flow in a meridional plane was recorded on video tape by illuminating the plane with a narrow strip (~ 0.5 mm) of light from an argon-ion laser.

When the homogeneous mixture of smoke and air was set into motion by rotating the bottom plate, a nonuniform distribution of flow markers developed, and within ~ 60 – 70 seconds a smoke-depleted steady vortex breakdown region was established. Figure 1b shows the steady state, with separation of smoke: the bright regions correspond to the presence and the dark regions to the absence of smoke. The separation process started in the near-axis region of the top surface and gradually propagated towards the bottom plate. In addition to the depletion of smoke in the near-axis region, a thin smoke-depleted layer was formed along the cylinder wall and top plate. This depleted layer, ~ 0.5 mm thick, was clearly visible to the eye, although it is not obvious in the pictures recorded. While clear (smoke-depleted) air was transported by the meridional flow into the top near-axis region, the depleted layer thickness along the wall increased, suggesting continual separation of smoke.

The separation phenomenon became more marked when the cylinder (along with the top plate) was rotated at an angular speed of $\Omega/2$ in the same direction as before. In this case, a vortex column free of smoke particles was formed on the axis. As in the preceding case, the darker segments initially appeared near the top plate, and then descended along the symmetry axis. An intermediate state and the final smoke-depleted vortex column are shown in Fig. 2a and b.

As the observed species separation is contrary to the accepted concept of continuum, we undertook careful checks to verify the phenomenon. To avoid accumulation of electrostatic charge which might cause near-wall particle depletion, the solid surfaces were treated with an antistatic solution. We illuminated

the flow cross-sections in several different horizontal and vertical planes, including some off axis. All cross-sections showed species separation. As an unambiguous test, we examined fluid samples collected from smoke-free and smoky regions during the experiment with a hypodermic needle; the results showed an absence of smoke in samples collected from a smoke-depleted region, consistent with the images.

The characteristic time for the nonuniform distribution of markers to be generated from the uniform initial state was fairly short, of the order of tens of seconds. This timescale is similar to that required for establishment of the steady hydrodynamical regime. An additional important feature is that the final smoke-depleted steady state is independent of initial conditions. Similar but less well defined separation was observed when dye was used in glycerin solution.

The observed separation has both theoretical and practical implications (for example in pollution control or isotope separation). Classical diffusion theory based on a continuum picture of the suspension fails to describe the observed separation. But as the number of particles is relatively small and they are massive compared to air molecules, for a tentative explanation of this kinematic separation we should instead consider the particles to be discrete and initially uniformly distributed. As the flow becomes established, the distribution of the particles becomes strongly nonuniform; furthermore, the particles avoid the walls of the vessel because of the development of a boundary layer. A more appropriate description of the phenomenon might be formulated in terms of lagrangian dynamics of the individual particles, rather than a continuum approach. We are optimistic that such separation of species may be possible not only for submicrometre-size suspended particles, but also for gases or liquids of different molecular weights. $\square$

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NMR characterization of isomers of C_{78} , C_{82} and C_{84} fullerenes

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FOLLOWING the development of a method for bulk synthesis of C_{60} and other fullerenes¹, the isolation of higher fullerenes ranging from C_{76} to C_{96} has been achieved using chromatographic techniques^{2–5}. Whereas C_{60} and C_{70} have unique, high-symmetry structures⁶, theoretical calculations for fullerenes larger than C_{76} have suggested that each may exist in at least two isomeric forms⁷. For C_{84} , 24 isomers have been postulated⁷, and for C_{96} calculations have yielded 196 distinct isomers⁸. Diederich *et al.*⁹ have used liquid chromatography and ^{13}C NMR to identify two isomers of C_{78} , but previous experimental studies of other higher fullerenes² have produced ambiguous results. Here we use ^{13}C NMR to determine the structures of some principal isomers of C_{78} , C_{82} and C_{84} . We find a third isomer of C_{78} , which was not reported in ref. 9. Characterization of the structures of these larger fullerenes should

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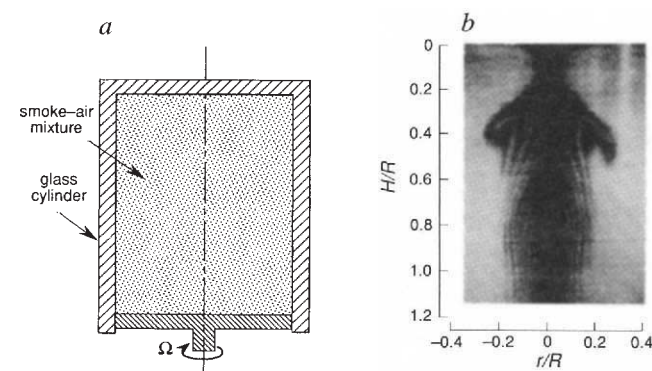


FIG. 1 a, Schematic of the flow facility. b, Flow visualization pictures showing the separation of smoke in vortex breakdown region.

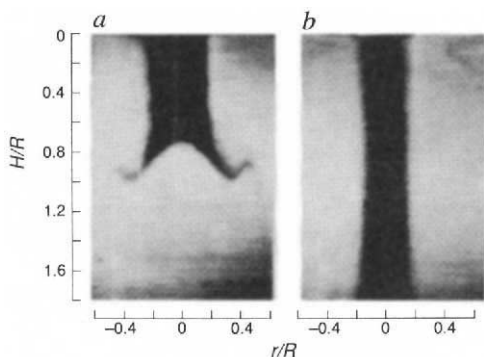


FIG. 2 Flow visualization showing a, an intermediate, and b, the final state of the smoke-depleted column.

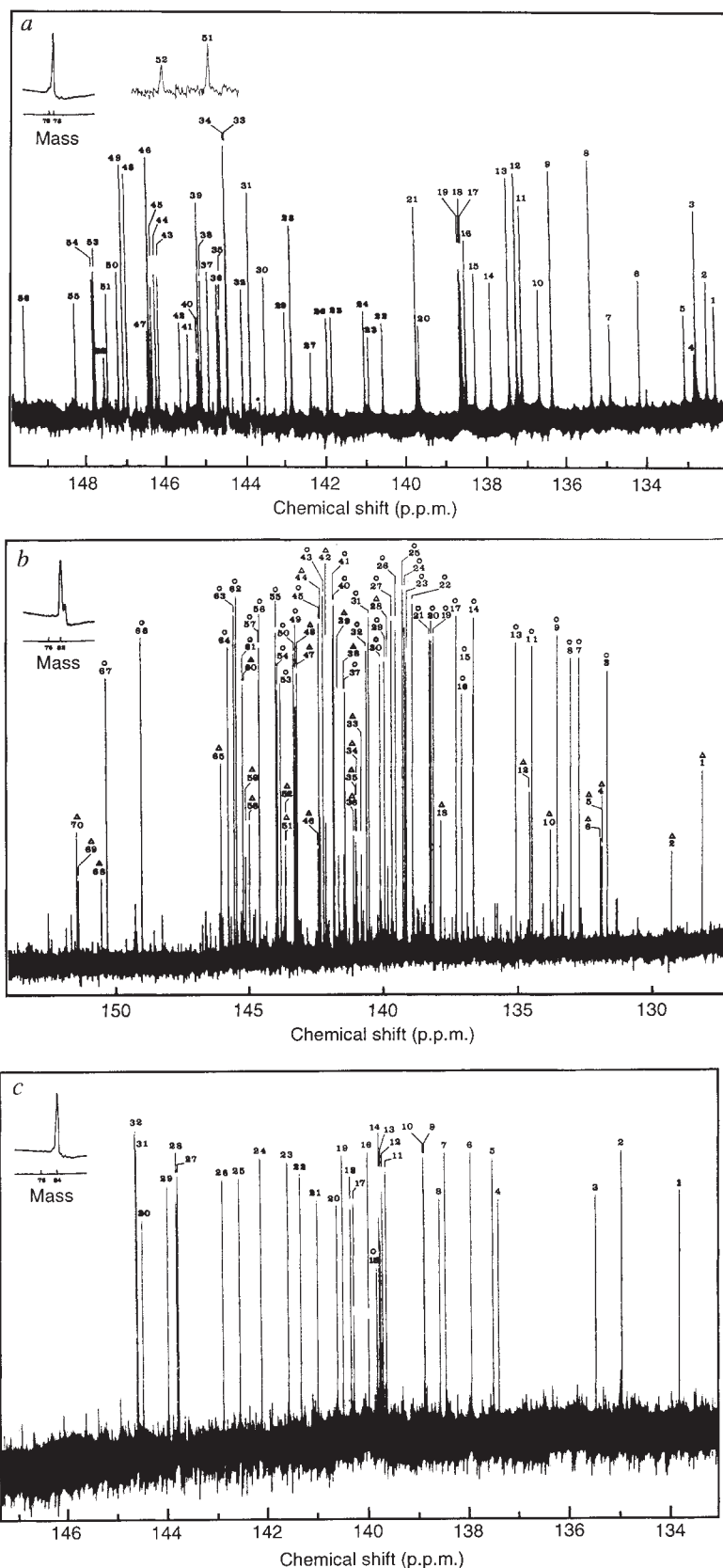


FIG. 1 ^{13}C -NMR spectra of fullerenes. *a*, C_{78} after 23,000 scans; *b*, C_{82} after 35,000 scans and C_{84} after 90,000 scans, in CS_2 solution, taken at 125.6 MHz, with acetone as an internal lock; $\circ$, strong lines, Δ , moderate lines. The region of lines 51 and 52 in *a* is expanded so that the linewidths can be compared; the different widths suggest differences in the spin-lattice relaxation rates of the carbon nuclei. Laser-desorption time-of-flight mass spectra are also shown for samples of C_{78} , C_{82} and C_{84} isolated by high-performance liquid chromatography. The line denoted 47 and 48 in *b* seems to be a single line with almost the same intensity as those of the strong lines ($\circ$). But throughout our two independent preparations and measurements of C_{82} , it was strongly suggested that the line arises from the overlap of two kinds of C_{82} isomers. One of its components was very sensitive to the experimental conditions. In one case (not shown here), the intensity of the line decreased to about half the intensity shown here. This reduction of intensity was accompanied by a decrease in eleven other lines, 1, 2, 5, 12, 18, 51, 52, 60, 65, 68 and 69. From the numbers of these NMR lines, it may be deduced that the component that is sensitive to the sample preparation is due to the isomer $\text{C}_{3v}\text{-C}_{82}$.

provide new understanding of the factors determining the stability of hollow carbon clusters.

We isolated C_{78} , C_{82} and C_{84} by high-performance liquid chromatography (the preparation is described in detail elsewhere^{4,5}). Laser-desorption time-of-flight mass spectra were obtained to confirm the purity of the isolated samples. We used an ArF (193 nm) laser as the desorption light source⁵. Mass

spectra for the samples of C_{78} , C_{82} and C_{84} are shown as inserts to Fig. 1a-c. We measured ^{13}C NMR spectra of the higher fullerenes using CS_2 as the solvent with $\text{Cr}(\text{C}_5\text{H}_7\text{O}_2)_3$ as a relaxant.

C_{78} . Figure 1a displays the ^{13}C -NMR spectrum of C_{78} , showing 56 distinct lines. From a recent paper by Diederich *et al.*⁶, one can easily identify the 21 lines (marked * in Table 1) due to

one of the two C_{2v} - C_{78} fullerenes out of the five fullerenes that satisfy the 'isolated pentagon rule' (IPR)¹⁰, which are slightly solvent-shifted by 0.17 p.p.m. Likewise one can easily identify the 13 lines (marked † in Table 1) that correspond to D_3 - C_{78} (solvent-shifted by ~0.09 p.p.m.). This leaves 22 peaks to be accounted for, 17 of which have relative intensity 3.9–6.0 and five have relative intensity 1.6–2.4 (Table 1). The other of the two C_{2v} - C_{78} candidates, corresponding to C'_{2v} in ref. 9, possesses 22 NMR lines with numbers of independent resonances $5 \times 2C$ and $17 \times 4C$. This situation is consistent with the present observation. Therefore, we can conclude that C_{78} consists of three isomers with symmetry C'_{2v} , C_{2v} and D_3 , in the ratio 5:2:2. The structures of the three isomers determined here are shown in Fig. 2a. Our conclusion is, however, inconsistent with that of Diederich *et al.*⁹, who showed the formation of two isomers for C_{78} , C_{2v} and D_3 , in the ratio 5:1.

C_{82} . The ^{13}C -NMR spectrum of C_{82} (Fig. 1b) consists of 41 strong lines, in addition to 29 lines of moderate intensity (the line marked 47, 48 looks like a single strong line, but in fact we believe it to be a superposition of two weaker lines; see Fig. 1b). Positions and intensities of the strong and moderate lines of C_{82} are summarized in Table 1. In Fig. 1b, we can also see at least 48 weak lines and more than 80 very weak lines. Here we note that the positions of some very weak lines coincide with the lines due to C_{84} described later. This evidence is consistent with the mass spectrum of C_{82} which shows it to be contaminated by a small amount of C_{84} .

According to Manolopoulos and Fowler⁷, C_{82} has nine IPR-satisfying isomers ($2 \times C_{3v}$, C_{2v} , $3 \times C_2$ and $3 \times C_s$), out of which the three isomers with C_2 symmetry give 41 NMR lines with nearly equal intensity. The 41 strong lines with intensities of 2.3–1.8 (Table 1) can therefore be easily assigned to the three C_2 - C_{82} . Three structural candidates for C_2 - C_{82} are shown in Fig. 2b.

On the other hand, C_{2v} - C_{82} and C_{3v} - C_{82} are expected to have combinations of ($17 \times 4C$, $7 \times 2C$) and ($12 \times 6C$, $3 \times 3C$, $1 \times 1C$ or $11 \times 6C$, $5 \times 3C$, $1 \times 1C$) lines, respectively⁷. Out of these 40 lines, 17 lines for C_{2v} and 12 lines (or 11 lines) for C_{3v} should be twice or six times as strong as the other lines. Therefore, if we assume that C_{2v} - C_{82} and one of the C_{3v} - C_{82} isomers are produced in almost equal amounts, the 29 observed lines with moderate intensity can readily be rationalized. The remaining nine weaker lines associated with these cages are considered to

TABLE 1 Line positions and relative intensities of the ^{13}C NMR spectra of C_{78} , C_{82} and C_{84} fullerenes

No.	C_{78}		C_{82}		C_{84}	
	Shift (p.p.m.)	Relative intensity	Shift (p.p.m.)	Relative intensity	Shift (p.p.m.)	Relative intensity
1	132.28†	2.3	128.20	1.0	133.81	1.7
2	132.48*	2.6	129.36	0.9	134.97	1.8
3	132.76	5.3	131.74	2.3	135.48	1.9
4	132.80*	1.1	131.93	0.7	137.39	1.6
5	133.04†	1.7	131.94	0.6	137.50	1.8
6	134.15*	2.8	132.00	0.7	137.94	1.6
7	134.88†§	1.9	132.78	2.2	138.46	1.4
8	135.36	4.9	133.10	2.1	138.57	1.7
9	136.34	5.0	133.60	1.9	138.88	2.0
10	136.66*	2.3	133.83	0.7	138.89	2.0
11	137.08	4.6	134.52	2.0	139.63	1.5
12	137.20	4.6	134.61	1.2	139.70	1.7
13	137.40	4.9	135.12	1.9	139.75	1.8
14	137.83*	2.6	136.68	2.0	139.77	1.5
15	138.23*	2.8	136.71	2.2	139.82	1.0
16	138.46	3.9	137.13	2.0	139.98	1.7
17	138.56*	2.2	137.34	2.2	140.27	1.6
18	138.58*	2.9	137.91	0.8	140.33	1.7
19	138.60	4.3	138.18	2.0	140.49	1.9
20	139.64†	1.6	138.27	1.8	140.61	1.3
21	139.71	4.6	138.33	2.0	140.99	1.5
22	140.55†	1.8	138.96	1.9	141.33	1.7
23	140.90†	1.6	139.19	2.1	141.58	1.7
24	141.00†	2.2	139.25	1.8	142.12	1.9
25	141.81§	2.1	139.32	2.4	142.55	1.8
26	141.92†	2.0	139.58	2.1	142.88	2.0
27	142.34*	1.5	139.72	1.9	143.77	1.8
28	142.82	4.6	139.88	0.6	143.80	1.7
29	142.97†	2.2	139.98	2.1	143.98	1.9
30	143.49*	3.0	140.15	2.1	144.48	1.9
31	143.86	4.9	140.56	2.3	144.58	2.2
32	144.06*	2.6	140.65	2.0	144.60	2.2
33	144.41	5.7	140.86	0.6		
34	144.43	6.0	141.02	0.6		
35	144.61†	2.0	141.06	0.7		
36	144.66*	2.7	141.14	0.7		
37	144.88*	3.0	141.45	2.3		
38	145.06*	2.5	141.50	0.7		
39	145.12	4.5	141.70	0.6		
40	145.17*	0.9	141.85	2.0		
41	145.39	1.6	141.88	1.8		
42	145.59†	1.6	142.17	0.8		
43	146.12*	2.6	142.25	2.2		
44	146.20*	2.8	142.38	0.9		
45	146.29*	2.5	142.41	1.9		
46	146.36	5.5	142.45	0.7		
47	146.40	2.4	143.23	0.7‡		
48	146.91	5.1	143.23	0.7‡		
49	147.02	5.0	143.29	2.0		
50	147.14*	3.0	143.36	2.0		
51	147.42	2.1	143.67	0.8		
52	147.51	1.6	143.69	0.8		
53	147.74*	2.8	143.85	2.2		
54	147.78*	2.5	143.97	2.0		
55	148.23†	2.3	144.02	1.9		
56	149.54†	2.0	144.65	2.5		
57			144.66	2.5		
58			145.03	1.1		
59			145.18	0.6		
60			145.27	0.8		
61			145.28	2.2		
62			145.52	2.3		
63			145.61	2.2		
64			145.83	2.1		
65			146.10	1.1		
66			149.04	2.4		
67			150.35	2.1		
68			150.56	0.6		
69			151.41	0.6		
70			151.47	0.7		

* Attributed to D_3 - C_{78} . † Attributed to C_{2v} - C_{78} .

‡ The intensity of the line 47, 48 was divided into two for each line.

§ An alternative explanation, suggested by R. Taylor (personal communication) is that these lines are due to C_{2v} and D_3 , respectively.

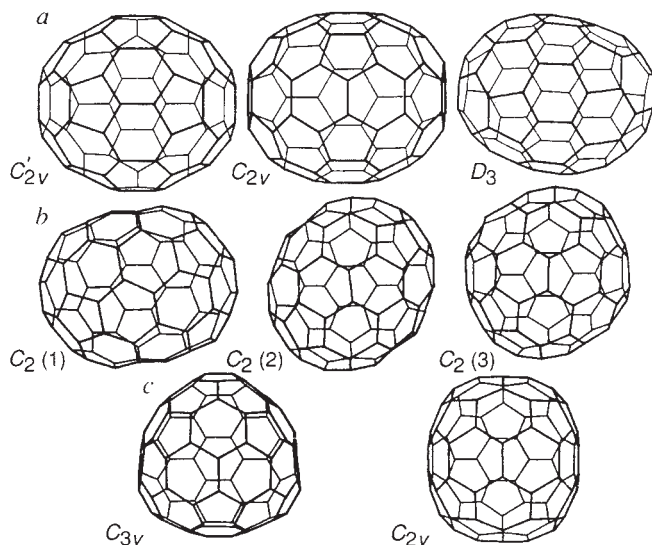


FIG. 2 The structures of fullerene isomers suggested by the ^{13}C -NMR measurements. a, Three isomers of C'_{2v} , C_{2v} and D_3 - C_{78} . b, Three structural candidates for C_{82} fullerene with C_2 symmetry. c, Structures of C_{2v} and C_{3v} - C_{82} isomers suggested by the ^{13}C -NMR measurements.

be hidden in the weak or very weak lines. In fact, we found that no other combination of C_{82} isomers could produce 29 NMR lines of nearly equal intensity. Quantitative analysis for the NMR lines with strong and moderate intensities indicates that the production ratios of C_{82} are 8:1:1 for C_{2-} , C_{2v-} and $C_{3v-}C_{82}$, respectively. The numbers of unidentified weak and very weak lines, on the other hand, suggest the existence of at least three more C_{2-} and/or $C_{5-}C_{82}$ isomers.

C_{84} . The first attempt to determine the structures of C_{84} was made by Diederich *et al.*². Possibly because of the low signal-to-noise ratios, their determination was not conclusive. The NMR spectrum of C_{84} obtained here is shown in Fig. 1c. Despite C_{84} being larger than the former two fullerenes, the NMR spectrum shows a much simpler profile, consisting of 31 strong lines and one additional line at half the intensity. Considering the predicted numbers and intensities of the NMR lines for C_{84} isomers⁸, we conclude that C_{84} has two major isomers. The most abundant isomer is one of the four possible D_2 cages and the other is the $D_{2d}-C_{84}$, which uniquely corresponds to the structure numbered 23 in Fig. 4 of ref. 8. The ratio of D_2 to D_{2d} is 2:1. This conclusion is surprising, because out of 24 candidates, only two isomers with relatively low symmetries are preferentially formed as a stable C_{84} fullerene.

These results provide some general insight into the formation and stability of higher fullerenes. Among many IPR-satisfying candidates, only limited numbers of isomers with, in general, low symmetry were found to be favoured in the carbon soot

generated by arc-heating of graphite. Furthermore, comparison of the present results with those previously reported strongly indicates that unidentified experimental factors in the sample preparation influence the stabilization of certain kinds of isomers as well as fullerenes. All of this experimental evidence suggests that growth processes may have an essential role in determining the structure of fullerene cages, rather than the structures being determined simply by thermodynamical stability considerations.

After this work was completed, we learned that Diederich and Whetten had reported 30 NMR lines at almost the same positions as ours¹¹. The formation of C_{82} has also been suggested to be sensitive to experimental conditions; no formation of C_{82} was seen by Diederich and Whetten¹¹ (see also ref. 2). □

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Consumption of atmospheric methane by desert soils

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ATMOSPHERIC concentrations of methane, a greenhouse gas, are increasing at a rate of about $1\% \text{ yr}^{-1}$ (refs 1–4). Oxidation by methylobacterial bacteria in soil is the largest terrestrial sink for atmospheric CH_4 , and is estimated to consume about $30 \times 10^{12} \text{ g CH}_4 \text{ yr}^{-1}$ (refs 4–6). Spatial and temporal variability in the rate of soil CH_4 consumption are incompletely understood^{6–19}, as are the apparent inhibitory^{12,13,18} or enhancing²⁰ effects of changes in land use. Dry deserts, which constitute 20% of total land surface, are not currently included in global soil uptake estimates. Here we describe measurements of the rate of uptake of atmospheric CH_4 by undisturbed desert soils. We observed rates as great as $4.38 \text{ mg CH}_4 \text{ m}^{-2} \text{ day}^{-1}$; 50% of the measured rates were between 0.24 and $0.92 \text{ mg CH}_4 \text{ m}^{-2} \text{ d}^{-1}$. Uptake of CH_4 by desert soil is enhanced by rainfall after an initial soil-drainage period—opposite to the response of temperate forest soils¹². Methane is consumed to a depth of about 2 m, allowing for deep removal of atmospheric CH_4 if near-surface conditions are unfavourable for consumption. On the basis of an annual average CH_4 consumption rate of $0.66 \text{ mg CH}_4 \text{ m}^{-2} \text{ d}^{-1}$, we estimate that the global CH_4 sink term needs to be increased by about $7 \times 10^{12} \text{ g yr}^{-1}$ to account for the contribution of desert soils.

We measured the uptake of atmospheric CH_4 by soils in the Mojave Desert/Southern Great Basin transition zone, ~150 km northwest of Las Vegas, Nevada. Measurements were made on 11 occasions between January 1989 and June 1991, in all months except April, July, August and November. Measurement locations included desert bajada (Jackass Flats), a treeless mountain crest (Yucca Mountain), wind-deposited sand ramps (Fran

Ridge) and a mountain crest in pinyon-juniper forest (Shoshone Mountain). Sparse vegetation typifies all sites; soils are predominantly sand overlain by ~10 mm of irregular rock fragments or gravel, except at Fran Ridge where slightly crusted sand is exposed at the surface. Table 1 summarizes physical and vegetational characteristics of the measurement locations.

We used the static chamber method^{22,23} to measure CH_4 uptake. Chambers were 0.25 m^2 in area and 0.2 m tall. Gas samples were collected at 10-min intervals for 1 h, in syringes having airtight stopcocks. Paired chambers, separated by ~10 m, were used at each location. Measurements were made in the same general area on each visit, repositioning chambers to avoid previously disturbed locations. CH_4 concentration was measured as a function of soil depth by withdrawing samples through stainless steel or nylon tubes installed to depths of 0.02 to 10 m. CH_4 analyses were done within 6 h of collection by gas chromatography flame ionization detection using an 80–100 mesh Porapak N column, 3.2 mm in diameter by 2 m long, and N_2 carrier. Uptake rates were calculated from linear regressions of the decay of CH_4 concentration in the chambers against time (mean $r^2 = 0.962$). The concentration was adjusted for prevailing temperature and atmospheric pressure according to the ideal gas law.

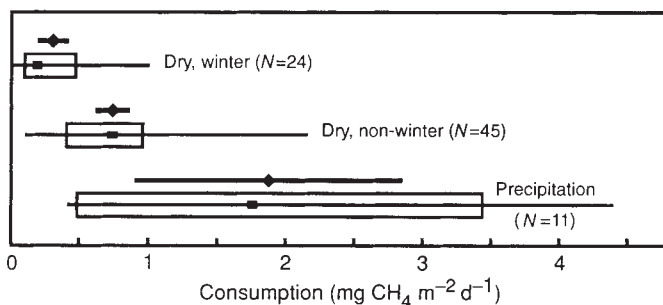


FIG. 1 Comparison of CH_4 consumption rates in desert soils for 'dry, winter', 'dry, non-winter' and 'precipitation' measurements. Upper lines are mean rates with 95% t -distribution confidence intervals. Lower box-plots are median rates with first and third quartile distributions and range of measured rates.

TABLE 1 Physical and vegetational characteristics of measurement locations

	Latitude; Longitude	Elevation (m)	Dominant vegetation ²¹	Soil organic matter (wt %)	Soil grain size (wt %)		
					>2 mm	0.063–2 mm	<0.06 mm
Jackass Flats	36° 50' N; 116° 16' W	1,100	<i>Larrea tridentata</i> <i>Atriplex confertifolia</i> <i>Ambrosia dumosa</i>	0.9	11	77	12
Yucca Mountain	36° 50' N; 116° 29' W	1,490	<i>Atriplex</i> sp. <i>Ephedra</i> sp.	2.9	18	70	12
Fran Ridge	36° 51' N; 116° 24' W	1,190	<i>Atriplex</i> sp. <i>Artemisia</i> sp.	0.6	8	90	2
Shoshone Mountain	37° 0' N; 116° 15' W	1,950	<i>Pinus monophylla</i> <i>Juniperus osteosperma</i>	2.7	17	53	30

Methane uptake rates for individual chambers in undisturbed soils ranged from 0 to 4.38 mg CH₄ m⁻² day⁻¹. Soil moisture in the 20–50 mm depth interval ranged from 0.56 to 11.6% by weight, and temperature at 50 mm ranged from 4.5 to 44.5 °C. Emission of CH₄ was never observed. Uptake was not consistently related to soil moisture and/or soil temperature at a fixed depth, but the measurements suggest that both antecedent precipitation and temperature influence CH₄ uptake.

Rates of CH₄ uptake among the four sites were not significantly different (ANOVA; $P=0.35$). We therefore analysed rates from all sites as a single group. Two subdivisions of the group were made. Measurements made within 10 days of the last observed precipitation were separated into a 'precipitation' group. The remaining 'dry' group was subdivided into 'dry, winter', represented by the months December, January and February, and 'dry, non-winter', represented by all other months. Figure 1 summarizes CH₄ uptake for these subdivisions; mean consumption rates for 'dry, winter', 'dry, non-winter' and 'precipitation', were 0.30 ± 0.26 , 0.74 ± 0.44 and 1.87 ± 1.45 mg CH₄ m⁻² day⁻¹, respectively. These rates suggest that consumption is inhibited by lower temperatures and enhanced following soil wetting. Uneven distribution of the physical and vegetational features of the desert soil surface contributes to the patchy distribution of consumption rate that is indicated by the relatively large standard deviations, particularly for recently wetted soils.

Enhancement of CH₄ consumption by increased soil moisture in dry soils has not previously been demonstrated, although an opposite, inhibitory effect for other soil types has been explained in terms of soil microbiology¹² and soil physics²⁴. The effect of precipitation on CH₄ consumption was tested by wetting experiments at Jackass Flats. Figure 2 shows averages of paired measurements as a function of time after 30 mm of water was applied on 20 October 1990 and 15 mm of water on 11 June

1991. In both experiments the initial effect was to inhibit CH₄ consumption because the water saturated the pore spaces. This was followed by increased consumption as the soil drained. The pre-irrigation consumption rate was approached after ~48 h for the October experiment and was maintained for 69 h. For this case, the combined inhibitory effects of reduced air-filled porosity due to water saturation, and reduced soil temperature caused by cooling of the wet soil apparently countered any enhancement of consumption that may have occurred during the observation period. Halving the water application, in June 1991, resulted in a 250% increase in CH₄ consumption after 48 h, from 0.41 to 1.03 mg CH₄ m⁻² day⁻¹.

The frequency of occurrence and the duration of the precipitation-induced enhancement are not precisely known, although they are important for estimation of the net desert consumption of CH₄. For example, the largest measured CH₄ uptake rate (4.38 mg m⁻² day⁻¹) occurred in May 1989, in two days following a five-day period during which 19 mm of rain fell. Only ~245 mm of precipitation fell during the entire 900-day study period, and precipitation totals greater than 15 mm were only exceeded during five precipitation periods having a total accumulation of 143 mm (ref. 25). If it is assumed that a year has ~3% 'precipitation', 72% 'dry, non-winter' and 25% 'dry, winter' days, the estimated mean annual rate of atmospheric CH₄ uptake for this desert area is 0.66 mg CH₄ m⁻² day⁻¹, comparable to uptake rates reported for grassland areas^{6,9,18}, yet less than rates for temperate forest soils^{12,16}.

The depth distribution of methylobacterial activity has importance in evaluating the influence of environmental factors such as soil moisture and temperature on atmospheric methane

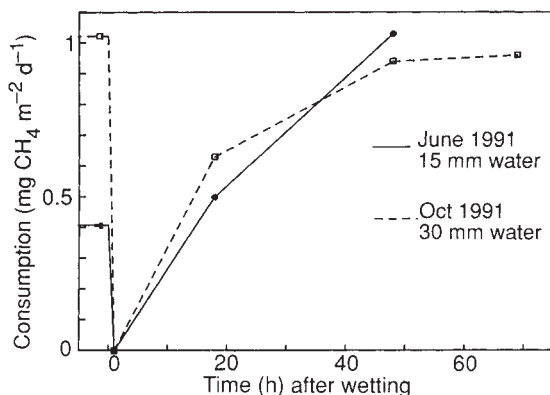


FIG. 2 Methane consumption rate against time following application of 30 mm water (October 1990) and 15 mm water (June 1991) at Jackass Flats. Condition before wetting is to the left of time 0. Zero flux at 1 h is due to water saturation of pore spaces at soil surface. Consumption rate increased >250% in 48 h for the June 1991 experiment.

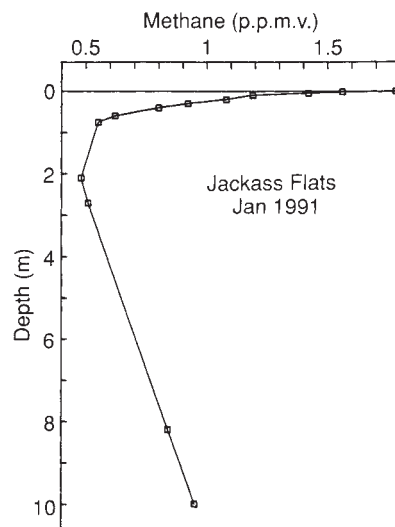


FIG. 3 Methane concentration, in parts per million by volume, against depth at Jackass Flats, January 1991. The general shape of the concentration profile is similar throughout the year.

uptake. Figure 3, showing CH_4 concentration against depth at Jackass Flats, indicates downward diffusion with net consumption of atmospheric CH_4 to a depth of ~2 m, coupled with upward diffusion without net consumption of a subsurface source of CH_4 to 2 m, where that CH_4 is also consumed. A similar 2-m-thick zone of atmospheric CH_4 consumption was predicted by numerical modelling of a grassland site in Illinois²⁴, but the thickness of such zones has not previously been verified by *in situ* measurement. This indicates that as long as a downward transport pathway is available, unfavourable conditions for methane consumption near the soil surface may allow atmospheric CH_4 to diffuse to some greater depth, where consumption can proceed under more favourable conditions. It also partly explains the inconsistent success of attempts to relate CH_4 uptake rate to soil moisture and/or temperature at a fixed depth near the surface. Precise measurements of subsurface change in CH_4 concentration and isotopic composition occurring in response to change in temperature, moisture, nutrient and physical conditions of soil are needed for better characterization of the gross CH_4 budget of all soil systems.

Our measurements indicate that desert soils are significant contributors to the global CH_4 soil sink term. They also suggest that the magnitude of CH_4 consumption by desert soils could increase substantially if there is a net increase in arid region soil moisture. Current general circulation models are, however, not capable of predicting with certainty any such regional

increases (or decreases) in soil moisture in response to the various global climate change scenarios²⁶. □

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Response of alluvial systems to fire and climate change in Yellowstone National Park

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PROJECTIONS of the ecological effects of global climate change often include increased frequency and/or intensity of forest fires in regions of warmer and drier climate^{1–3}. In addition to disturbing biological systems, widespread intense fires may influence the evolution of the physical landscape through greatly enhanced sediment transport⁴. Debris-flow to flood-streamflow sedimentation events following the 1988 fires in the Yellowstone National Park area (Wyoming and Montana, USA) have allowed us to examine the geomorphological response to fire in a mountain environment. Abundant analogous deposits in older alluvial fan sequences bear witness to past fire-related sedimentation events in northeastern Yellowstone, and radiocarbon dating of these events yields a detailed chronology of fire-related sedimentation for the past 3,500 years. We find that alluvial fans aggrade during periods of frequent fire-related sedimentation events, and we interpret these periods as subject to drought or high climatic variability. During wetter periods, sediment is removed from alluvial fan storage and transported down axial streams, resulting in floodplain aggradation. The dominant alluvial activity is strongly modulated by climate, with fire acting as a drought-actuated catalyst for sediment transport.

Fire regimes in the high-elevation conifer forests of the Yellowstone region are characterized by large, infrequent, stand-

replacing events, as exemplified by the fires of 1988 (ref. 5). We have studied the effects of such fires on the northeastern Yellowstone landscape (Fig. 1a), an area of steep-walled glacial trough valleys cut in highly erodible Eocene volcanoclastic rocks. Small tributary basins, many with ephemeral streams, feed postglacial (paraglacial⁶) alluvial fans along the base of main valley side-slopes. In burned basins, loss of vegetative cover and addition of combustion residues to soil surfaces result in large increases in storm runoff as overland flow^{4,7}. Beginning in summer 1989, charcoal-rich debris flows have been generated by brief, intense convective-storm precipitation in small, steep, intensely burned basins with little postfire regrowth. Fine sediment, ash and charcoal entrained by sheetwash and rilling of burned soil surfaces combine with coarser sediment derived from channel incision to produce the high sediment concentration of these flows. Larger basins tend to produce more-dilute newtonian-flood streamflows, and hyperconcentrated flows (transitional from streamflow to debris flow)^{8,9}. Runoff from a single storm commonly progresses towards more dilute flow but may also be dominated by a single process. Since 1989, events have been less frequent, mostly because of revegetation, and more dilute, probably because of processes that decrease fine sediment availability even on unvegetated slopes¹⁰. Instability persists, however, because deep incision accelerates ravelling and slumping into channels of event basins.

We examined deposits produced by a range of post-1988 fire-related sedimentation processes to develop models for identification of similar deposits within alluvial fan sequences in northeastern Yellowstone. In debris flows, large clasts settled and were emplaced in bouldery lobes where flows became unconfined below the alluvial fan apex. Fines-dominated run-out slurries continued downfan, depositing thin, widespread lobes of very poorly sorted muddy sand with abundant charcoal and organic material (Fig. 1b). Similar charcoal-rich debris-flow deposits are common in northeastern Yellowstone fan sequences, and provide diagnostic records of fire-related sedimentation which can be radiocarbon-dated.

Charcoal remains suspended in more-dilute flows and is therefore rare within fire-related hyperconcentrated-flow and streamflow fan deposits. Many alluvial fan stratigraphic sections in

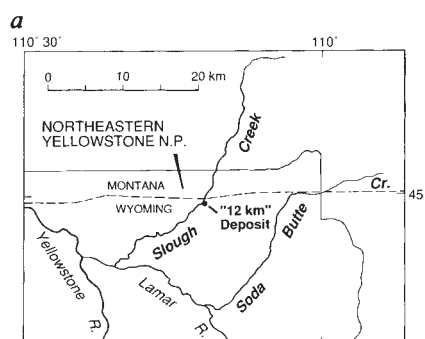
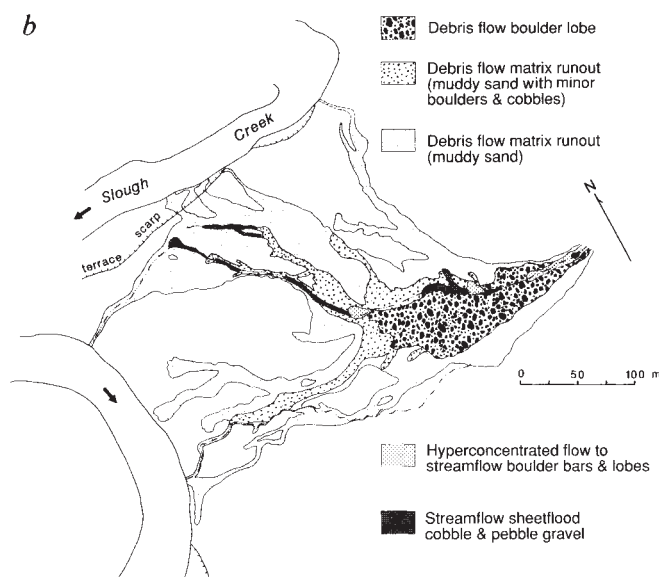


FIG. 1 *a*, Map of the northeastern Yellowstone area, showing the Soda Butte and Slough Creek drainages, where dating of Holocene fire-related and other alluvial deposits was done. Because of intense burns covering entire low-order basins, the steeper areas of Yellowstone have generated a number of large fire-related sediment transport events from 1989 to 1991 (refs 28, 29). One example is the '12 km' event (centre) of July 1989, dominated by debris flow; deposits of this event are shown in *b*. Fines- and charcoal-rich run-out facies cover ~40% of the alluvial fan, which is roughly outlined by the deposits. These facies are also prevalent in Late Holocene stratigraphic sections of alluvial fans.



Yellowstone, however, contain thin, burned-in-place soil surface layers. Preservation of these charcoal-rich layers without bioturbation requires rapid burial; post-1988 fire-related sediments have buried an intact 1988 burned surface in several localities. Using this analogue, we interpret hyperconcentrated-flow and streamflow facies units directly overlying well preserved burned-in-place layers as probable fire-related deposits (less confidently interpreted as fire-related than are charcoal-rich debris-flow deposits). Data from 25 stratigraphic sections in northeastern Yellowstone show that fire-related debris-flow and probable fire-related sediments make up ~30% of the volume of late Holocene alluvial fan materials. A substantial part of the remaining volume is likely to be reworked or nondiagnostic fire-related sediment. Fire has been an important factor in sediment transport from slopes and low-order channels into storage on alluvial fans.

We identified and radiocarbon-dated 18 fire-related debris-flow events and 17 probable fire-related sedimentation events spanning the past 3,500 years in the Soda Butte and Slough Creek drainages of northeastern Yellowstone (Figs 1 and 2). Data on earlier Holocene events (not shown) are relatively sparse because of limited exposure. Tree-ring calibrated¹¹ ages are plotted in the histogram in Fig. 2; where multiple calibrated

ages are possible for one radiocarbon date, the date is plotted in the interval containing the greatest area underneath the calibrated probability density distribution (CPDD) for that date. The normal distribution defined by the ¹⁴C date and its analytical standard deviation is transformed to a CPDD through the calibration curve in the computer program CALIB¹¹. Probability density curves in Fig. 2 are constructed by summing individual CPDDs for the same 35 dates shown in the histogram, where each date contributes an equal portion of the area under the curve. The highest frequency of variation in the curve largely represents multiple possible calibrated ages for single events (compare histogram and curve). The curve is truncated at AD 1872 because historical records and stratigraphic relations constrain the youngest fire-related event to before that date. The peak near AD 1700 probably represents the last widespread fires in Yellowstone, as recognized in ~350-yr dendrochronological^{5,12} and lake sediment¹³ fire histories.

Although most dated samples from fire-related sediments were aggregates of charcoal fragments, we obtained five dates on individual charcoal fragments from one debris-flow unit (Fig. 2). Distributions for four of these dates strongly overlap, implying that aggregate sample dates primarily reflect one age of charcoal: that from the associated fire.

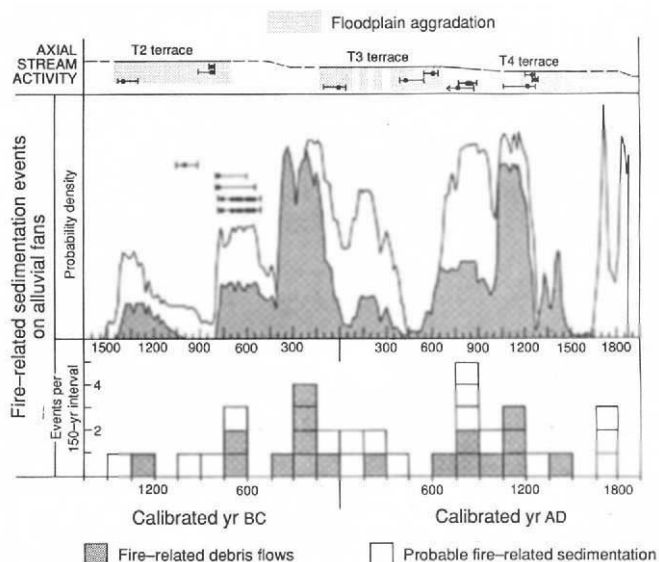


FIG. 2 Summary of fire-related sedimentation and other alluvial activity in the Soda Butte and Slough Creek drainages for the past 3,500 years. Histogram and probability density curves show the distribution of calibrated ¹⁴C dates¹¹ (in calendar years) on 35 independent events interpreted as either fire-related debris flows or probable fire-related sedimentation events (see text). Solid circles and error bars above the left end of the curve depict calibrated age(s) and 1 σ calibrated age ranges for five dated charcoal fragments from a single fire-related debris-flow unit; only the youngest date is included in the curve. Top of diagram shows time of activity on the T2, T3 and T4 axial stream terraces, including floodplain aggradation (patterned bars) and downcutting between levels (sloping lines), as constrained by associated calibrated ¹⁴C dates (solid circles and error bars). Symbols are dashed where activity is uncertain. Date with arrow on error bar ($\leftarrow$) denotes a minimum limiting age for T3 floodplain aggradation, and date directly above represents a local time of downcutting to T4 level.

Fire-related alluvial fan sedimentation events show strong clustering within the intervals of 800 BC–AD 350 and AD 650–1250, and sediment accumulation rates indicate concurrent rapid aggradation of the northeastern Yellowstone fans. Records of non-arboreal pollen¹⁴ and the relative abundance of xeric- and mesic-adapted small mammals¹⁵ in northern Yellowstone indicate that effectively drier summer conditions prevailed over these intervals. Maxima of fire-related debris-flow activity occur around 350–100 BC and AD 1000–1200 (Fig. 2); the latter maximum occurs within the widely recognized Mediaeval Warm Period (~AD 900–1300¹⁶ with optimum at ~AD 1090–1230^{17,18}). It thus seems that fire-related sedimentation is most active during centennial-scale drought-dominated periods (Fig. 3b).

Our analyses of historical records (1895–1989) of climate and fires in Yellowstone show that 36% of the temporal variance in annual burn area is explained by drought-related surface climate variables for the concurrent summer and preceding winter¹⁹. Because large fires may occur with short, severe drought (1–2 yr), and subsequent intense precipitation is also required to mobilize sediment, an alternative (but not exclusive) hypothesis is that fire-related sedimentation is generated by high short-term climate variability. The relatively low precision of ¹⁴C ages makes this possibility difficult to evaluate. Coincidentally, evidence for high decadal to interannual variability combined with anomalous warmth is seen in tree-ring series from the western United States^{20,21} and Fennoscandia²² around AD 1160, corresponding roughly with a major peak in fire-related debris-flow activity in northeastern Yellowstone (Fig. 2).

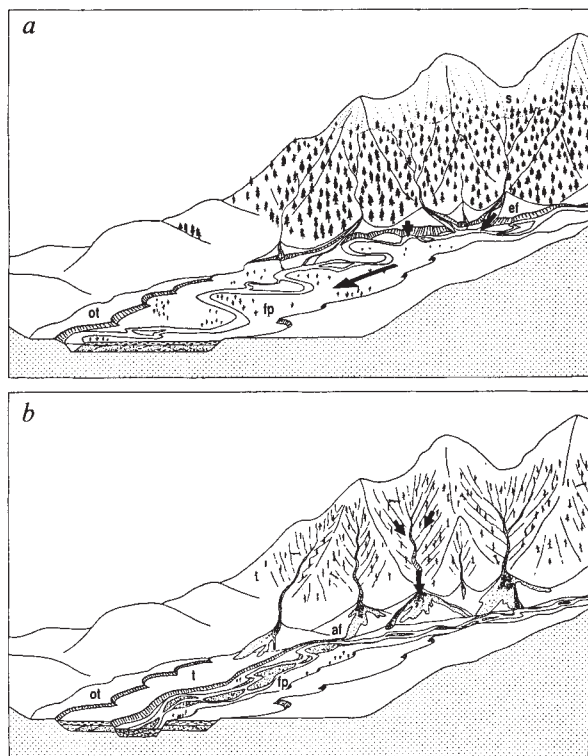


FIG. 3 Sketches of idealized Holocene alluvial activity modelled after Soda Butte Creek, with arrows denoting dominant modes of sediment transport; exaggerated for clarity. *a*, Activity during wetter periods, characterized by high snowmelt runoff (s), erosion of alluvial fans (ef) by fan channel incision and fan toe undercutting, and floodplain (fp) widening and aggradation along the lower axial stream, where sideslope sediment input is low. An older terrace (ot) remains from a previous period of floodplain aggradation. *b*, Activity during drier periods, with recently burned slopes supplying abundant sediment for debris-flow transport to aggrading alluvial fans (af). The axial stream is incising within a narrow active floodplain (fp), leaving the former wet-phase floodplain of *a* as a terrace (t).

We obtained calibrated ¹⁴C dates on Soda Butte and Slough Creek axial stream sediments, from a variety of stratigraphic settings and sample types (Fig. 2). Although the late Holocene trend in lower Soda Butte Creek has been one of downcutting, this trend has been interrupted by periods of floodplain widening and aggradation which are recorded in terrace deposits. Each of these periods corresponds to a minimum of fire-related fan sedimentation. Floodplain deposits of the T3 and T4 terraces began aggrading shortly after major episodes of fire-related sedimentation, probably as these sediment loads reached downstream locations²³. The relatively low fire-related activity preceding T2 floodplain aggradation, however, implies that fluctuations in hydroclimate and annual snowmelt runoff (which produces most of the water and suspended sediment discharge in these streams at present²⁴) may more effectively control axial stream activity than slope-derived sediment supply. Local palaeoenvironmental data^{14,15} characterize periods of floodplain aggradation as effectively wetter, particularly the T3 floodplain aggradation period culminating around AD 350–650 (Fig. 2). In this same period, we have also found evidence for large-scale slumping of previously stable Pleistocene deposits, greatly increased activity of a travertine-depositing spring and an increase in channel sinuosity in lower Soda Butte Creek. These changes are consistent with higher groundwater levels and increased frequency of small overbank floods. Sharply declining fire-related sedimentation and early T4 floodplain construction following AD 1200 are coincident with onset of the Little Ice Age elsewhere in North America^{18,21,25}. Although a large pulse of fire-related sedimentation precedes this period, floodplain widening and aggradation of the T4 terrace is relatively minor, and is more consistent with the slight increase in effective precipitation due to cooling implied by local palaeoenvironmental data^{14,15}. With increased average stream power in wetter periods, the dominant alluvial activity switches to removal of sediment from alluvial fan storage and downstream transport. Increased sediment discharge causes temporary reversal of the downcutting trend in lower reaches of axial streams, and floodplain aggradation results (Fig. 3a).

Low-amplitude climate changes were sufficient to induce considerable adjustments in these alluvial systems. Despite regional variations in the nature of late Holocene climate change and resultant geomorphological response, the timing of alluvial episodes in northeastern Yellowstone is similar to that in other parts of the western United States²⁶. More gradual response to climate variations may occur through vegetation change in the absence of fire, but late Holocene changes in forest/grassland ratio and vegetation density occurred mainly at lower elevations in Yellowstone^{14,27}. Such changes probably had limited effects on high-elevation alluvial systems²⁶, and were subordinate to the major slope erosion and fan deposition caused by short-term severe drought and temporary, total devegetation by fire. □

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Reactivation of an oceanic fracture by the Macquarie Ridge earthquake of 1989

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THEORETICAL calculations show¹ that the occurrence of an earthquake leads to regions of increased shear stress on both sides of the ruptured fault. If this stress increase is sufficiently large, or if neighbouring faults are close to their failure stress, the result may be triggering of earthquakes on pre-existing faults in the region. A search for this effect² produced only a few examples, all in continental areas, where clusters of aftershocks were located in the regions of increased stress. The linear dimensions of these clusters were generally less than 20 km, and less than one-half of the fault length that ruptured in the main shock. Here I report an example of this phenomenon in which the fault that is reactivated is a nearly 175-km-long segment of an oceanic fracture, and

a 23 May 1989 – 31 December 1989

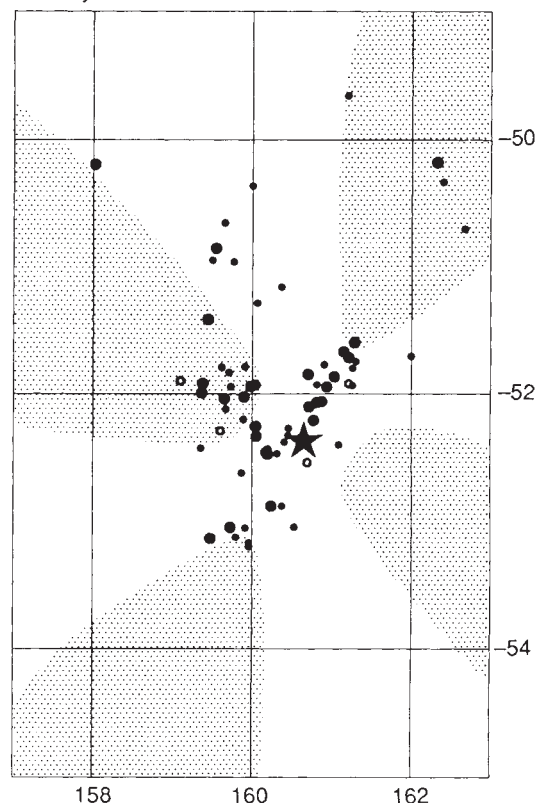


FIG. 2 a, Relocated aftershock epicentres for all events within the box in Fig. 1 during the period from 23 May 1989 to 31 December 1989, indicated by solid circles. The Macquarie Ridge main shock is plotted as a star. Larger symbol sizes indicate larger events. The 79 aftershocks had magnitudes in the range given by $3.8 \leq m_b \leq 5.6$, with 10 of them being too small for ISC to be able to assign a magnitude to them. These events are indicated by open circles (one size). Aftershocks are seen to concentrate on two linear zones, one being the India/Australia–Pacific plate boundary and the other being an elongated feature to its west. Events with $m_b \geq 5.0$ generally have location errors of less than a few kilometres, with the error decreasing with increasing earthquake magnitude. The four small aftershocks to the east of the Macquarie Ridge have large errors of location in the east–west

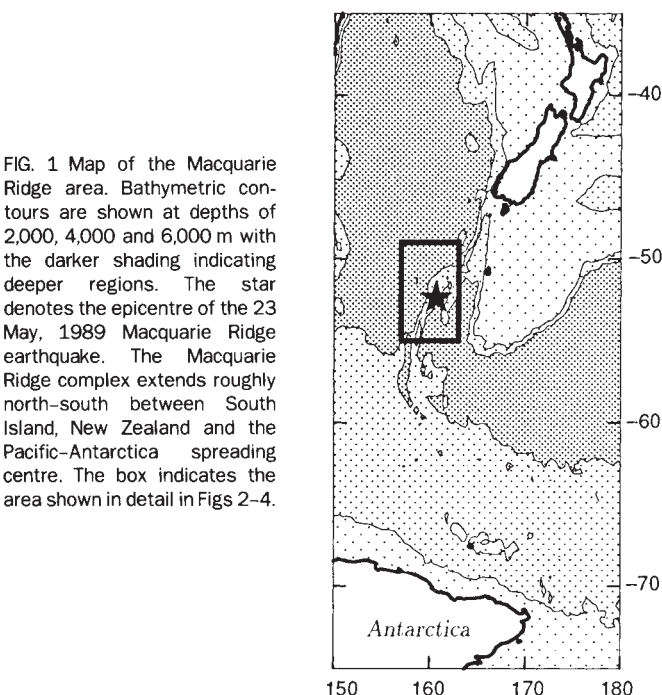
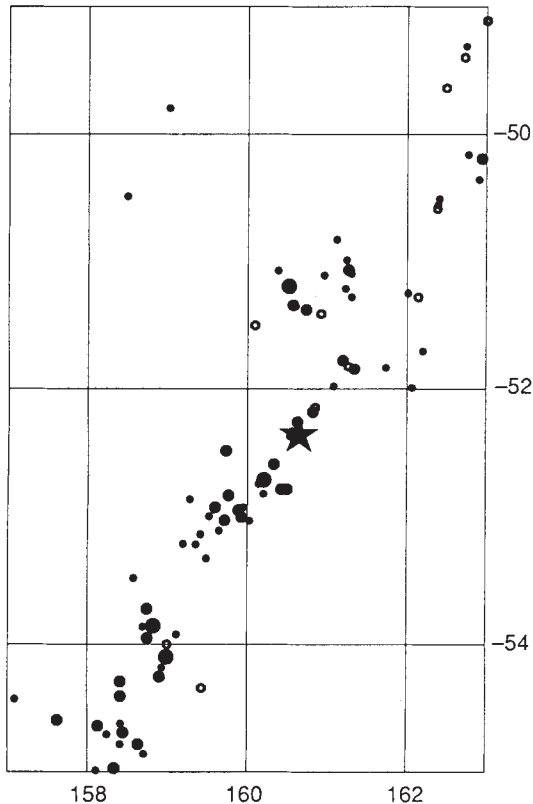
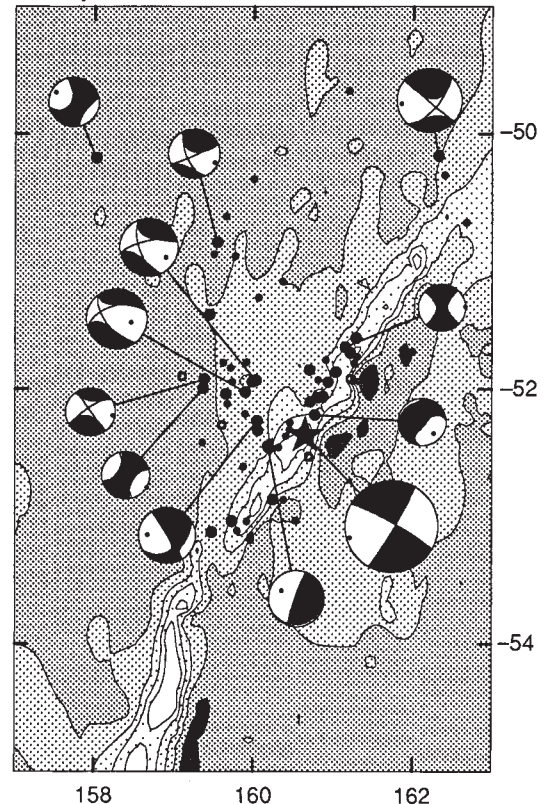


FIG. 1 Map of the Macquarie Ridge area. Bathymetric contours are shown at depths of 2,000, 4,000 and 6,000 m with the darker shading indicating deeper regions. The star denotes the epicentre of the 23 May, 1989 Macquarie Ridge earthquake. The Macquarie Ridge complex extends roughly north–south between South Island, New Zealand and the Pacific–Antarctica spreading centre. The box indicates the area shown in detail in Figs 2–4.

its length is comparable to that of the main shock, the 1989 Macquarie Ridge earthquake.

The 23 May 1989 Macquarie Ridge earthquake ($M_w = 8.2$), with a seismic moment 1.34×10^{21} N m (ref. 3), was the largest seismic event to have occurred globally for ~12 years and the largest on the India/Australia–Pacific plate boundary south of New Zealand in more than 70 years (Fig. 1). The main shock has been the subject of several studies^{4,5}. From readings of arrival times at worldwide stations compiled by the International Seismological Centre (ISC), all events near this earthquake for which at least seven stations reported *P*-wave readings between 1 January 1964 and 31 December 1989 were relocated using the method of joint hypocentre determination (JHD89)⁶. Figure 2a shows the aftershock distribution and Fig. 2b the seismicity from 1964 until the day before the main shock. Figure 3a shows the aftershocks with their Harvard centroid moment tensor (CMT) solutions^{7,8} superimposed on the bathymetry, and Fig. 3b the same for the 25-year period preceding the event (from quarterly listings⁹ of CMT solutions since 1977).

The aftershock map shows that there are two linear zones along which the aftershocks are concentrated. One is a 220-km segment of the India/Australia–Pacific plate boundary on which the main shock occurred^{3–5}. The other is a linear feature to its

b 1 January 1964 – 22 May 1989*a* 23 May 1989 – 31 December 1989

directions suggesting that they occurred on the ridge itself. Stippling indicates the regions of increased shear stress predicted for a vertical strike-slip fault corresponding to the main event. Although the regions are those predicted for a highly idealized, planar fault in an infinite homogeneous elastic medium, it is expected that the corresponding regions in the case of the Macquarie Ridge event would be of similar geometry and orientation, and that complications due to the free surface, nonplanar fault surface and so on would not strongly affect the conclusions. *b*, Same as *a* but for the 25-year period preceding the event. The main shock is also shown as a star. The linear feature to the west of the plate boundary which was activated by this earthquake (*a*) is seen to be seismically quiet during this period.

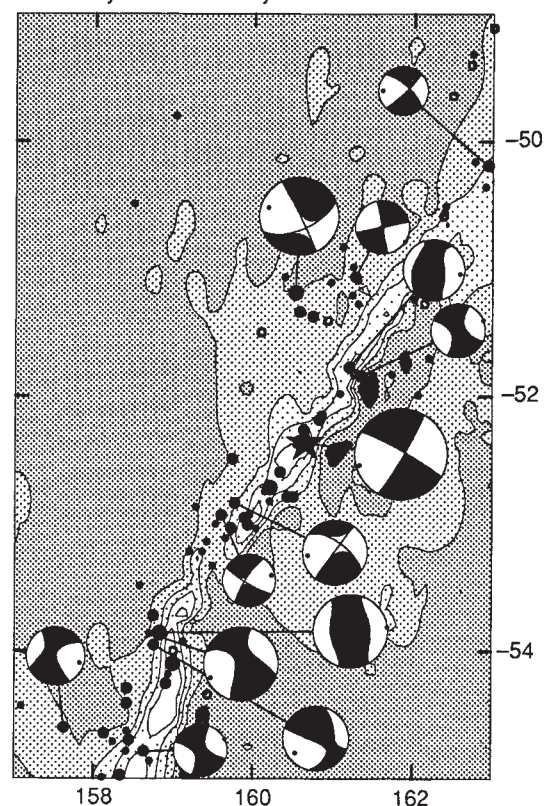
b 1 January 1964 – 22 May 1989

FIG. 3 *a*, Seismicity with the available Harvard CMT solutions for aftershocks^{7,8}. The size of the CMT solution symbol is related to its seismic moment. For the main shock, shown by the star, the CMT solution is replaced by the solution obtained from broad-band data³. Contours of the bathymetry at 1,000-m intervals to 6,000 m are shown, with the darker colour indicating deeper regions. The main shock is seen to be a pure right-lateral strike-slip with the shocks in the elongated zone of activity to its west being primarily left-lateral strike slip. I have redone the CMT solutions for the two largest events on this second feature to confirm that the small dip of the fault plane was not an artefact of the CMT method. For a pure strike-slip mechanism with the same strikes as in the CMT solution, the fits of the data to the model were significantly poorer (the r.m.s. error of fit was increased) confirming that this dip is indeed real. The dip of this plane explains why the aftershock distribution along this feature is diffuse when seen in map view. There is no significant aftershock activity in the region of off-fault stress increase to the east of the ridge suggesting either that the sea floor there is less fractured or that if it is fractured, these faults are far from their failure stress and are not triggered by the main earthquake. *b*, Same as *a* but with 25 years of seismicity before the event and CMT solutions from 1977 on⁹. The main shock is shown as a star, together with its CMT solution. In the 30-day period preceding the main shock, there was an event of $m_b=5.2$ on 9 May 1989 at the relocated position of $(-52.9, 159.6)$. It is thus located at one end of the main shock rupture and is at the edge of the region identified here as a 'geometric barrier'. The solutions in the 25-year period show that the background seismicity along

the central portion of the Macquarie Ridge is primarily right-lateral strike-slip. It also shows an earthquake of $M_s=7.0$ (refs 9, 13) which occurred at $(-51.2, 160.3)$ on 7 July 1982 and its aftershocks.

west, trending in the northwest-southeast direction, nearly 175 km long and lying close to the 4,000-m bathymetric contour. An examination of the original ship track data for bathymetry, although sparse, shows that the sea floor to the west of this feature is deeper and relatively smooth. In the area to the east and up to the Macquarie Ridge, the sea floor is shallower and rougher, suggesting a discontinuity in the nature (for example, the age) of the sea floor on the two sides of the 4,000-m bathymetric contour. The background seismicity for the 25-year period preceding the event (Fig. 2b) illustrates the fact that this linear feature was not active before the earthquake, the first aftershocks appearing on it ~2.5 hours after the main shock.

For an earthquake of its size, the Macquarie Ridge event had relatively few and small aftershocks. In the seven-month period following the earthquake, 79 aftershocks were reported, 44 occurring within the first seven days and only 17 in the five months since 1 August 1989. Twenty-nine of these were associated with the linear feature to the west of the plate boundary. Moreover, the largest aftershock ($m_b = 5.6$) of moment 1.0×10^{18} N m (ref. 7) occurred ~38 hours later, not on the main fault plane but on this second feature. Only 12 events were large enough for CMT solutions^{7,8} to be reliably obtained and of these, three of the smallest were on the main fault. These numbers contrast with those for the 1986 earthquake in the

Andreanof Islands, Alaska⁹⁻¹¹ ($M_w = 8.0$) with a seismic moment of 1.0×10^{21} N m, where nearly 150 aftershocks large enough to be relocated occurred within 24 hours of the earthquake and 238 events of $m_b \geq 4.7$ occurred within the nine-month period after the earthquake of which 36 were large enough for CMT solutions to have been obtained. Considering earthquakes of $m_b \geq 5.0$ only, there were 32 aftershocks in the seven-month period after the Macquarie Ridge earthquake compared with 88 in the same period after the Andreanof Islands event.

The CMT mechanisms in Fig. 3a show that the rupture of the main fault plane was pure right-lateral strike-slip. For the second feature, assuming that the fault plane is the nodal plane with strike similar to the trend defined by the aftershocks, the motion is seen to be primarily left-lateral strike slip. The magnetic anomaly data for this area (Fig. 4a) show that there is a left-lateral offset of magnetic anomalies 12, 13 and 18 on the two sides of a northwest-southeast feature at this place. The trend of the seismically defined feature follows the trend of a fracture zone inferred from this magnetic anomaly data¹² and is parallel to other fracture zones to its west. This fracture lies largely within the zone of increased off-fault shear stress that is expected to the west of the ridge for this earthquake¹, indicated in Fig. 2a.

Figure 4b shows the tectonic interpretation of the earthquake

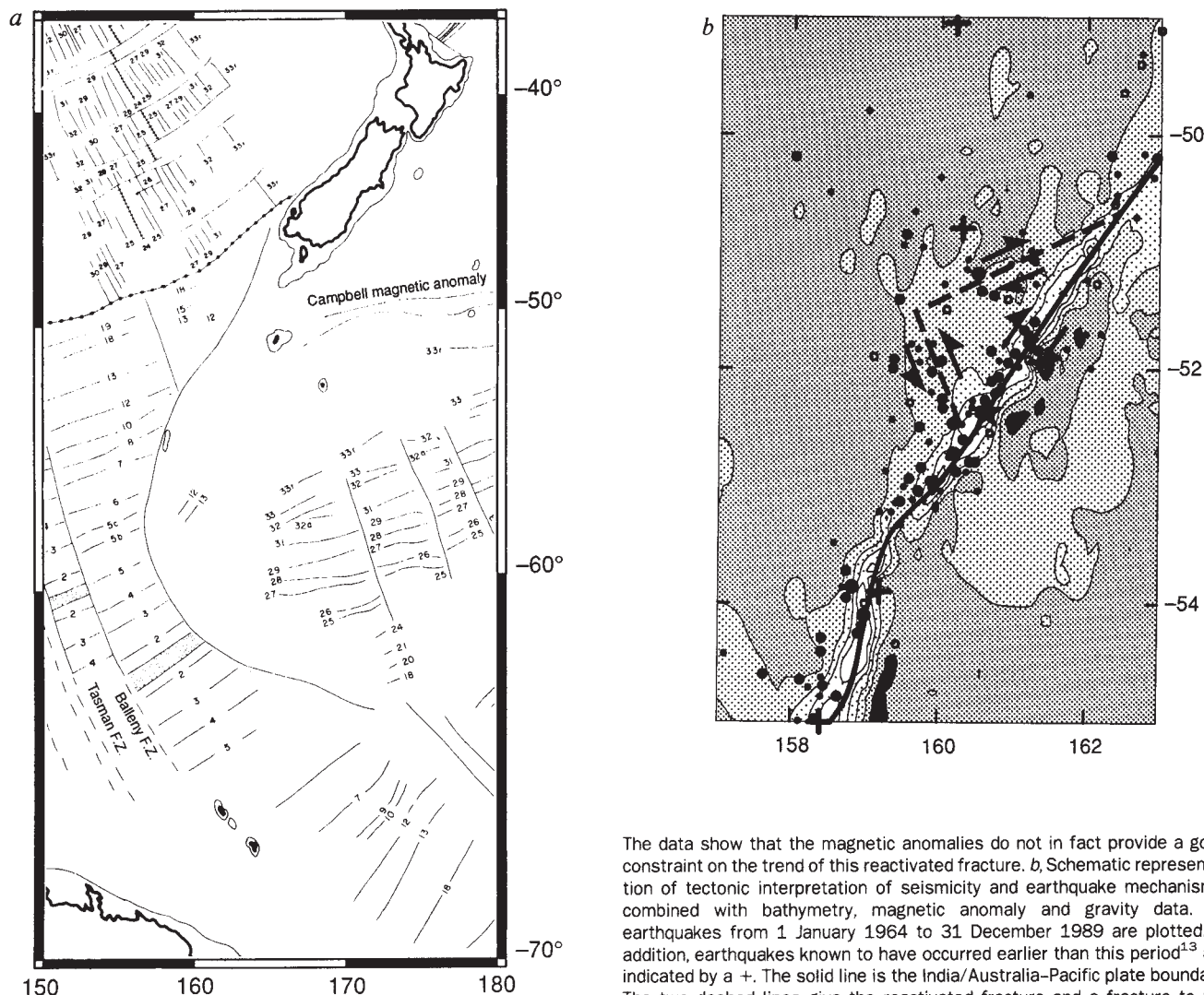


FIG. 4 a, Magnetic anomaly map of the area based on the data of Cande *et al.*¹². Lines with numbers are magnetic anomaly lines. Lines without numbers are plate boundaries or fracture zones inferred from the anomaly data¹².

The data show that the magnetic anomalies do not in fact provide a good constraint on the trend of this reactivated fracture. b, Schematic representation of tectonic interpretation of seismicity and earthquake mechanisms, combined with bathymetry, magnetic anomaly and gravity data. All earthquakes from 1 January 1964 to 31 December 1989 are plotted. In addition, earthquakes known to have occurred earlier than this period¹³ are indicated by a +. The solid line is the India/Australia-Pacific plate boundary. The two dashed lines give the reactivated fracture and a fracture to the north on which an earthquake of $M_s = 7.0$ occurred in 1982 (ref. 13). The small aftershocks to the north of this triangle suggest that this reactivated fault continues northwards but may have a change in trend. As only one mechanism in this place is available, the interpretation is not carried further.

mechanisms combined with the locations of all known events in the area. It suggests that the triangular area between the plate boundary, the reactivated fracture and a fault to the north on which an earthquake of magnitude ($M_s = 7.0$) occurred in 1982 (refs. 9,13; Fig. 3b) is being 'squeezed' towards the north and west. This triangular feature is clearly seen in a map of the anomaly in free-air gravity¹⁴.

Finally, the Macquarie Ridge earthquake is the clearest example I know of termination of an oceanic fault rupture by a 'geometric barrier'¹⁵. The aftershocks on the main fault end very abruptly in the south in a cluster, which is located at the point where the bathymetry shows a clear break in the Macquarie Ridge. The ridge is offset to the west by more than 20 km here and the trend changes from north-northeast to the north of this break to nearly north-south to the south of this break. □

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Rates of mitochondrial DNA evolution in sharks are slow compared with mammals

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THE rate of mitochondrial DNA (mtDNA) evolution has been carefully calibrated only in primates¹. Similarity between the primary calibration and rates estimated for other vertebrates^{2–4} has led to widespread assumption of a constant molecular clock in vertebrates even though this has never been rigorously tested⁵. We report here the examination of mtDNA sequence variation for 13 species of sharks from two orders that are well represented in the fossil record to test the constancy hypothesis. Nucleotide substitution rates in the cytochrome *b* and cytochrome oxidase I genes in sharks are seven- to eightfold slower than in primates or ungulates. This difference in substitution rate cannot be explained by nucleotide composition bias, codon-usage bias, selection, or choice of genes sequenced, and was confirmed by comparing species recently separated by the rise of the Isthmus of Panama. Such

TABLE 1 Regression analysis of the number of transversions per site against origination and first appearance time for lineages of sharks and primates

Group	Rate ($\times 10^{-10} \text{ yr}^{-1}$)	r^2	95% CI ($\times 10^{-10} \text{ yr}^{-1}$)	Rate through the origin ($\times 10^{-10} \text{ yr}^{-1}$)
Origination times				
Carcharhinoids only*	6.3	0.86	2.8, 9.9	7.2
Lamnoids only†	8.2	0.84	3.2, 13.3	8.1
All sharks‡	7.1	0.86	5.0, 9.1	7.8
First appearance times (from Fig. 3a)				
All sharks*	7.0	0.68	3.6, 10.0	9.2
Primates†	64.7	0.90	25.4, 100.0	50.1

Primate analysis was based on the ND4 and ND5 genes¹ using the same methods as for sharks (see Fig. 2 legend). Macaque was used to root the least-squares topology for primates. Origination and first appearance times are given in Fig. 1 for sharks and in Fig. 3a legend for primates.

† $P < 0.05$; * $P < 0.01$; ‡ $P < 0.001$.

differences in mtDNA substitution rates among taxa indicate that it is inappropriate to use a calibration for one group to estimate divergence times or demographic parameters for another group. High-resolution studies of molecular evolutionary rates require taxon-specific calibrations.

Using the polymerase chain reaction (PCR), sections of 855–1,551 base pairs (bp) from the cytochrome *b* and cytochrome oxidase I protein-coding genes were amplified and sequenced from 13 species of sharks representing 12 distinct lineages. To minimize *a priori* the influence of selection in measurements of molecular differences, we focused on substitutions at 4-fold degenerate sites. We first considered only transversion substitutions because they accumulate linearly over time⁴ and thus are relatively free from saturation effects^{6,7}.

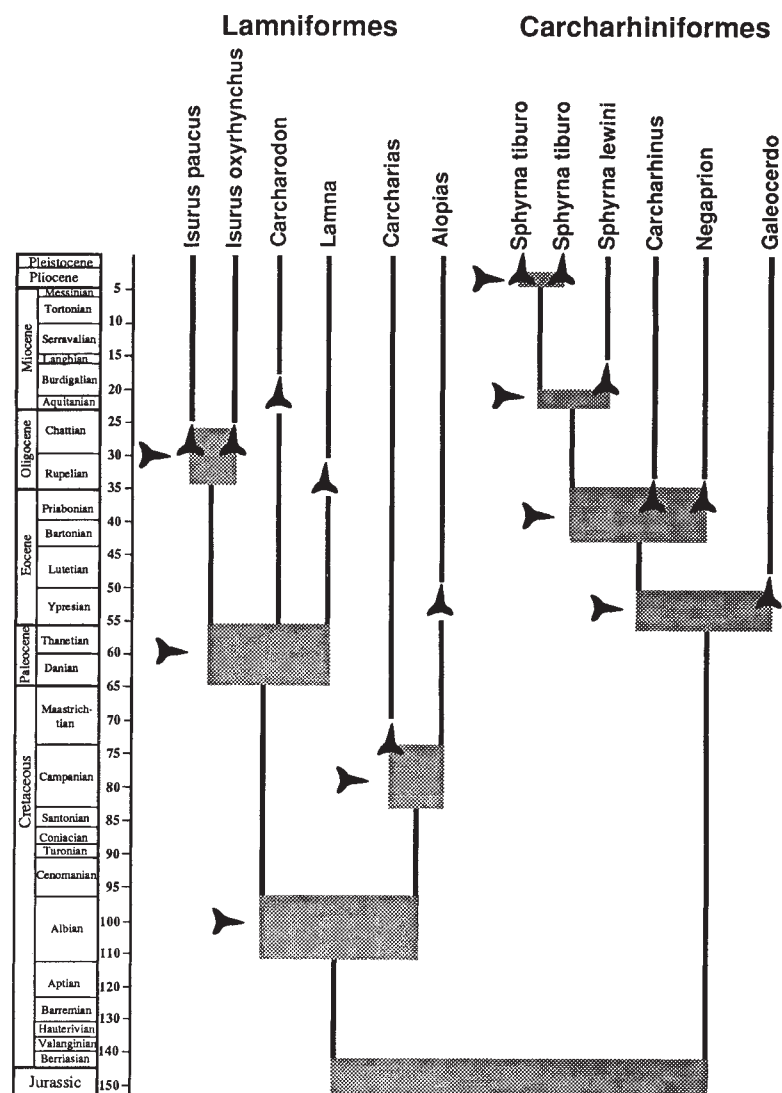
First appearance times of each lineage were compiled from the known fossil record (Fig. 1). From these data, we estimated evolutionary rates in two ways. First we used the first appearance of a taxon in the fossil record as the minimum time for evolutionary change along that lineage. Second, we overlaid the inferred topology of relationships from phylogenetic analysis of mtDNA sequence data on the stratigraphic record of first appearance times (Fig. 1). Origination and divergence time of taxa were taken to be the oldest fossil occurrence of either lineage. The inferred phylogeny and the available fossil record are largely concordant.

We estimated rates of nucleotide substitution in two ways. First, we estimated the number of transversion changes along each lineage (Fig. 2) and plotted these values against first appearance in the fossil record for that lineage (Fig. 3a) and against origination times. Substitution rates estimated from first appearance and origination times are similar for the two orders of sharks and are at least five times slower in sharks than in mammals (Table 1). Second, we plotted the average corrected number of transversion differences between species against divergence times (Fig. 3b). Divergence rates estimated in this way are about six- to sevenfold slower in sharks than in ungulates or primates.

To evaluate whether transitions also show a slower rate of accumulation in sharks, we compared complete cytochrome *b* sequences from two individuals of populations of the bonnethead shark (*Sphyrna tiburo*) separated by the Isthmus of Panama. Migration of these warm-water marine sharks between the Pacific and Atlantic is unlikely either around the tip of South America or through the freshwater Panama canal. Thus, the two populations were probably separated about 3.5 Myr ago⁸. The bonnetheads are 8% different (corrected for multiple hits⁹) at fourfold sites, suggesting a maximum divergence rate of 2.3% per million years. By comparison, chimpanzee and human mtDNAs differ by 27% at silent sites after about 5 Myr divergence, giving a divergence rate of 5.4% per million years.

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FIG. 1 Phylogenetic relationships among extant sharks and the stratigraphic record of first appearance times based on the occurrence of distinctive fossil teeth. Represented taxa are *Carcharhinus porosus* (smalltail shark), *Negaprion brevirostris* (lemon shark), *Galeocerdo cuvieri* (tiger shark), *Sphyrna lewini* (scalloped hammerhead), *S. tiburo* (bonnet-head), *Isurus oxyrinchus* (shortfin mako), *I. paucus* (longfin mako), *Carcharodon carcharias* (white shark), *Lamna nasus* (porbeagle), *Alopias superciliosus* and *A. vulpinus* (bigeye and common thresher, respectively), and *Carcharias taurus* (sand tiger shark). Phylogenetic relationship among species in each order was established using maximum-likelihood analysis and least-squares cluster analysis of the transversion differences between taxa (see Fig. 2) using the computer program PHYLIP³⁰. On the basis of maximum-likelihood analysis, branch lengths not much different from zero were collapsed. The inferred topologies of relationships in each group are corroborated by previous hypotheses based on analysis of morphology^{23,24} and isozymes²⁵. Upright shark teeth identify the first appearance of lineages in the fossil record. Sideways shark teeth identify minimum origination and divergence times based on the inferred phylogenetic relationships. First appearance times (in millions of years ago) and references are as follows: *Isurus oxyrinchus* and *I. paucus*, ≥ 30 (ref. 26); *Carcharodon*, 20–23 (ref. 27) (however, the *Carcharodon* lineage may have originated from *Paleocarcharodon* in the Paleocene^{27,28}); *Lamna*, 30–35 (ref. 27) (however, *Lamna* may have originated from *Cretolamna* sometime in the Late Cretaceous–Early Paleocene^{27,28}); *Alopias*, 50–56 (refs 26, 28) (Alopiidae is also known from the Cenomanian, 90–97 Myr, although this date is uncertain²⁷); *Carcharias* (= *Odontaspis*), 74–83 (ref. 27); *Sphyrna*, 20–23 (ref. 27); *Carcharhinus*, Middle Eocene 38–50 (ref. 27); *Negaprion*, Middle Eocene 38–50 (ref. 27); *Negaprion*, Middle Eocene 38–50 (ref. 27); *Galeocerdo*, 50–56 (ref. 27). The divergence between *Sphyrna*, *Carcharhinus*, and *Negaprion* occurred by Late Eocene time¹⁰ (38 Myr). Origination times for the two lineages of *Sphyrna tiburo* assume that the divergence of these geminate taxa coincided with the rise of the Isthmus of Panama in the Pliocene⁹. The divergence time between *Alopias* and *Carcharias* is based on the first appearance date for *Carcharias*, although the divergence may be more ancient than this.



A similar rate is obtained for ungulates based on comparison of sheep and goat. These data confirm that sharks have a slower mtDNA substitution rate than primates and ungulates.

The reduced rate of mtDNA evolution in sharks could be an artefact of an incomplete or misinterpreted fossil record. First occurrences of a taxon in the fossil record will only underestimate its age; therefore Fig. 3a provides a maximum rate of change consistent with the data. Yet this maximum is still

substantially lower than in mammals. To bring our data into line with the mammalian rate would require that first appearance times for 12 distinct lineages from two orders of sharks are overestimated by five to seven times. Because the fossil record of shark teeth is abundant and reasonably continuous, such systematic bias is unlikely¹⁰. Voluminous fossil, biochemical and molecular data¹¹ suggest that the primate data are also correct.

FIG. 2 Topologies of the inferred relationships among carcharhinoid (a) and lamnoid (b) sharks. The topologies were constructed from matrices of uncorrected transversion differences per 4-fold degenerate site using the FITCH algorithm in PHYLIP³⁰. Branch lengths are given. (Similar branch lengths were obtained when we used the neighbour-joining algorithm.) Topologies for each order were rooted using species from the other order as an outgroup, but branch lengths were determined for each order independently of the other. The branch length for the *Alopias* lineage is the median of two species, *Alopias superciliosus* and *A. vulpinus*. Primer sequences and protocols for the extraction, amplification and sequencing of DNA are available in ref. 31. Sequences and EMBL accession numbers available from A.P.M.

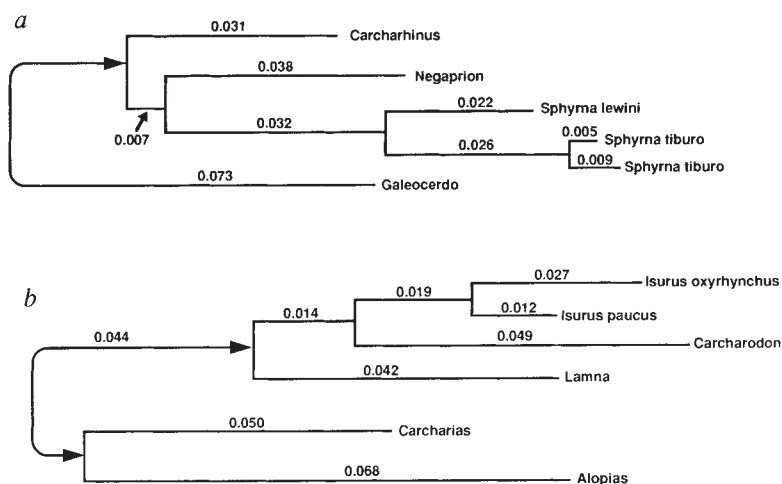
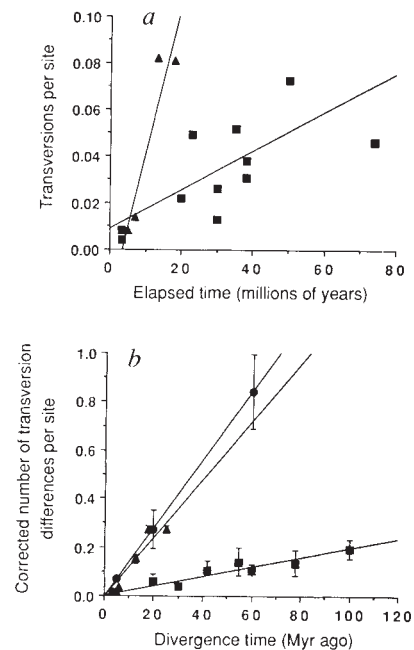


FIG. 3 *a*, Number of transversions per 4-fold degenerate site in independent shark (■) and primate (▲) lineages plotted against first appearance times of the lineages. Number of transversions per site per lineage estimated using least-squares analysis (see Fig. 2 and legend). First appearance times for sharks are from Fig. 1. Regression statistics are given in Table 1. All primate data are from ref. 1 and using first appearance and divergence times of 5 Myr, chimp against human; 6 Myr, gorilla against chimp/human; 13 Myr, orangutan against hominoids; 18 Myr, gibbon against great apes; 25 Myr, macaque against higher primates. *b*, Graph of the average corrected number of transversion differences per 4-fold degenerate site in pairwise comparison against divergence time (divergence times for sharks from Fig. 1). Error bars are ± 2 s.d. of the mean from all pairwise comparisons. ●, Ungulates; ▲, primates; ■, sharks. Corrected numbers of transversions per site were estimated using the equation, $K = 0.5 \ln(1/1 - 2Q)$ (ref. 9), where Q is the proportion of transversions. Ungulates are represented by the divergences between sheep and goat (5 Myr), cow versus deer/sheep/goat (20 Myr), and Suidae versus Ruminantia (60 Myr)⁴. The regressions are through the origin and yielded average evolutionary rates of 70, 60 and 10×10^{-10} transversions per 4-fold degenerate site per million years for ungulates, primates and sharks, respectively.



Molecular divergence in sharks could be slow, despite a high substitution rate, if constraints on nucleotide composition are more severe in sharks than in mammals¹². Nucleotide bias can be estimated as $B = \sum |0.25 - f_i| / 1.5$, where f_i is the frequency of the i th base at 4-fold degenerate sites. Sharks and primates have similar biases: average (± 1 s.d.) B values are 0.42 ± 0.07 and 0.41 ± 0.04 , respectively, suggesting that the reduced rate in sharks is not due to enhanced compositional constraints. Codon usage patterns are also similar, with an average of 3.7 different codons used across all 4-fold degenerate codons in the sequences from sharks and mammals. Furthermore, because we are sampling 4-fold degenerate sites, it is unlikely that the rate differences are the result of differences in selection or are an artefact of the genes sequenced and compared¹³.

Various hypotheses have been proposed to explain rate heterogeneity in nuclear genes, including differences in generation time¹⁴, DNA repair efficiency¹⁵ and exposure to mutagens¹⁶. Sharks¹⁷ and primates have similar generation times, indicating that this hypothesis may not extend to mitochondrial genomes. If rate heterogeneity reflects differential repair efficiency, then perhaps mammals have lost a major repair pathway or sharks possess replication machinery with remarkable fidelity. Finally, mtDNA damage *in vivo* is proportional to metabolic rate¹⁸ because oxygen radicals are potent intracellular

mutagens¹⁹. The evolutionary implication of these measurements is that taxa with low weight-specific metabolisms might show slower mtDNA evolution. The metabolic rate in shark tissues is 5–10 times lower than in mammals of similar body size²⁰. Reduced mtDNA evolution suggested for other poikilothermic taxa^{21,22} provides additional, emerging support for a link between metabolic physiology and mtDNA evolution.

The rate discrepancy between sharks and mammals has important implications for the comparative use of molecular data in systematics, population biology and molecular evolution. First, estimates of divergence times based on molecular differences and an inappropriate calibration will be wrong. Here the error would be almost 10-fold. Second, estimates of genetically effective population size are based on a comparison of observed genetic diversity and the expected rate of change. If the wrong calibration is used, estimates of effective population size and inferences of the demographic history of populations could be severely in error. Third, efforts to identify physiological, life-history or environmental effects on evolutionary rates must be based on careful calibration of these rates in different taxa. Our demonstration of a rate difference in sharks can be considered as a first step in the elucidation of such effects on rates and patterns of vertebrate mtDNA evolution. □

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Myosin head movements are synchronous with the elementary force-generating process in muscle

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MOTOR proteins such as myosin, dynein and kinesin use the free energy of ATP hydrolysis to produce force or motion, but despite recent progress^{1–4} their molecular mechanism is unknown. The best characterized system is the myosin motor which moves actin filaments in muscle^{5–12}. When an active muscle fibre is rapidly shortened the force first decreases, then partially recovers over the next few milliseconds⁵. This elementary force-generating process is thought to be due to a structural 'working stroke' in the myosin head domain^{5–9}, although structural studies have not provided definitive support for this^{10–12}. X-ray diffraction has shown that shortening steps produce a large decrease in the intensity of the 14.5 nm reflection arising from the axial repeat of the myosin heads along the filaments^{13,14}. This was interpreted as a structural change at the end of the working stroke, but the techniques then available did not allow temporal resolution of the elementary force-generating process itself. Using improved measurement techniques, we show here that myosin heads move by about 10 nm with the same time course as the elementary force-generating process.

When a small shortening step lasting less than a millisecond is applied to an active muscle fibre the force decreases during the step, then partially recovers over the next few milliseconds^{5,15}. The force decrease reflects the simple elastic behaviour of the myosin heads attached to actin filaments^{15,16}, and the rapid recovery is thought to result from nearly synchronous execution of the elementary force-generating process in the attached heads⁵. The process can be rapidly repeated by applying a series of shortening steps¹⁷. When steps corresponding to filament sliding of about 6 nm are repeated at 20-ms intervals, a steady state is attained in which rapid force recovery of about 80% of that in the first step is followed by a small slower phase (Fig. 1a). Thus the force-generating process is largely reprimed in the 20 ms interval between steps¹⁷.

We used this protocol to study the structural basis of force generation by X-ray diffraction. The intensity of the 14.5 nm X-ray reflection from single muscle fibres was measured with 200- μ s time resolution by signal averaging the step responses in a series. The time course of filament sliding (the sarcomere length change Fig. 1a, b), was measured simultaneously in the region of the fibre illuminated by the X-rays. The intensity of the 14.5-nm X-ray reflection decreases with about the same time course as the rapid force recovery, the elementary force-generating process (Fig. 1b). There is no detectable change in intensity during the length step itself and the accompanying elastic force decrease. Thus the myosin head movements underlying the intensity change do not result from filament sliding *per se* and do not passively follow changes in force; they are associated only with active force generation.

After the elementary force-generating process the intensity of the 14.5-nm axial reflection recovers with a half-time of about 8 ms for a 6-nm step (Fig. 1c). This is similar to the half-time for recovery of the capacity to repeat the elementary force-generating process, which is about 5 ms in comparable conditions¹⁷. Thus both the onset and recovery time courses of the

myosin head movements match those of the elementary force-generating process. The recovery is likely to result from rapid detachment from actin of myosin heads which have generated force, followed by reattachment in a state capable of doing so again^{13,17}. The change in the intensity of the 14.5-nm X-ray reflection shown in Fig. 1c agrees roughly with previous data at 1 ms time resolution obtained by applying 1-ms-length steps to whole muscles¹³. Although the rapid force recovery and the time course of the X-ray intensity decrease were not resolved in that study, it was estimated that the change in X-ray intensity lagged the fall in force by about 0.5 ms (compare with Fig. 1c).

In conventional models of muscle contraction^{5–9}, the elementary force-generating process involves a working stroke in which

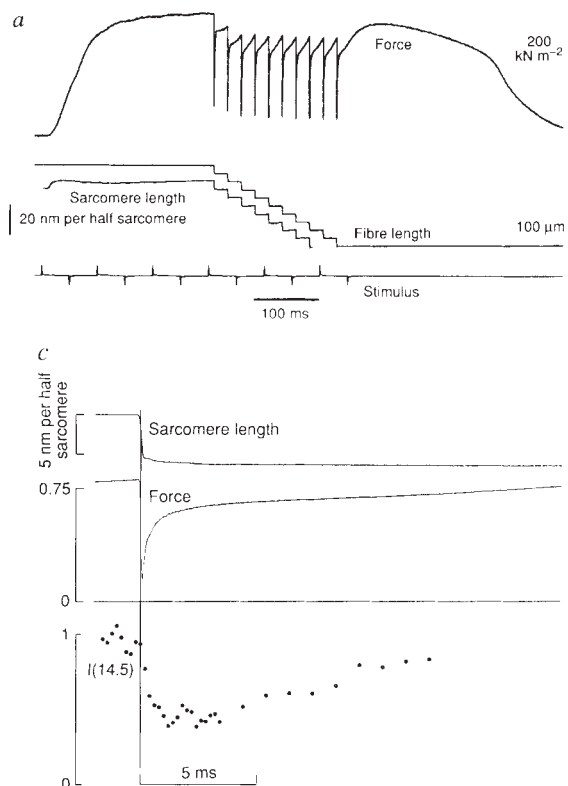
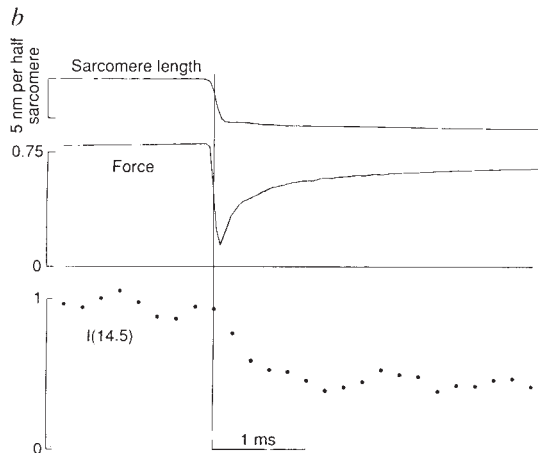


FIG. 1 Changes in force and the intensity of the 14.5-nm X-ray reflection resulting from a 'staircase' length change in an active single muscle fibre. **a**, Slow timebase records of force and length changes. Fibres were stimulated at constant length to allow development of maximum isometric force before imposition of a series of 10 shortening steps, each of duration 0.12 ms and resulting in filament sliding of about 6-nm per half sarcomere, at 20-ms intervals. A steady-state force response, comprised of an elastic force decrease, a rapid partial recovery and a smaller slow recovery, is attained after the first 2–3 steps. The sarcomere length record is truncated at the point where axial fibre movement accompanying shortening led to the loss of the striation follower signal. Cross-sectional area of fibre, 35,700 μ m²; initial fibre length, 6.27 mm; initial mean sarcomere length in the 1.4-mm segment interrogated by the X-ray beam, 2.22 μ m. **b**, Fast timebase records of sarcomere length, force and the intensity of the 14.5-nm X-ray reflection, $I(14.5)$, showing the average response to 6-nm steps in a series of 10 steps at 20-ms intervals, as in **a**. The sarcomere length and force traces are averaged from five fibres. The force record shows the elastic decrease and rapid partial recovery phases of the response to the shortening step. The X-ray intensity data, recorded in 0.2-ms time frames, are from three of these fibres and are normalized by the mean value before the step. In the other two fibres the X-ray data were recorded at 2-ms time resolution. The mean (±s.e.m.) decrease in the intensity of the 14.5-nm reflection after the step in the five fibres was 53 ± 4% of its value before the step, similar to that in the three fibres in the figure. The intensity before the step was 9 ± 4% of that in a tetanus without imposed steps; this is related to the small cumulative reduction in intensity during the series of 10 steps accompanying that in force. The vertical line denotes the midpoint of the length step; the X-ray data point on this line represents the 0.2-ms time frame.

myosin heads move by about 10 nm, generating force by stretching a structurally distinct elastic element^{5,9}. Each working stroke is assumed to be coupled to the hydrolysis of one ATP molecule. This type of model has been challenged by reports that actin filaments can slide by more than 60 nm per ATP molecule hydrolysed^{18,19}, although this result is controversial²⁰⁻²². The large decrease in the intensity of the 14.5-nm X-ray reflection now shows directly that myosin heads move by a substantial fraction of 14.5 nm during the elementary force-generating process, as predicted by conventional models with a 10-nm working stroke. This does not rule out a total sliding distance per ATP hydrolysed which is much bigger than 10 nm, because mechanical¹⁷ and structural (Fig. 1c) data show that the working



during which the length step was completed. c, As in b but with a slower timebase to show the recovery of the intensity of the 14.5-nm reflection (at 0.2 and 1.0-ms intervals) after the step.

METHODS. Intact single fibres were isolated from anterior tibialis muscles of the frog *Rana temporaria*. The tendons were trimmed and mounted in aluminium foil clips¹⁵ to attach one end of the fibre to a moving-coil motor^{24,27} and the other end to a capacitance gauge force transducer²⁸. Average sarcomere length in the region of the fibre illuminated by the X-ray beam was measured with a striation follower²⁹. Fibres were stimulated to give fused tetani by transverse alternating-polarity pulses from platinum plate electrodes. The temperature was 3–4 °C. Details of the mechanical arrangements have been described previously²⁴. Station 2.1 at the SERC Synchrotron Radiation Source (Daresbury, United Kingdom), with a monochromator/mirror X-ray camera³⁰ was used to provide an intense X-ray beam focussed to 2.5 × 0.3 mm at the fibre. Slits further reduced the beam size along the fibre to about 1.5 mm to match the segment in which sarcomere length was measured. X-ray exposure was limited to the period of data acquisition by a fast shutter. After about 20 s total exposure, fibres became inexcitable because of radiation damage; up to this time mechanical and X-ray responses were stable. The X-ray path through the bathing solution was reduced to about 0.7 mm by placing Kapton windows close to the fibre. The X-ray pattern was recorded on a two-dimensional detector and associated data acquisition system³⁰ with a 4.3-m camera. Data analysis used computer facilities and the BSL and OTOKO packages provided by SERC Daresbury Laboratory. The intensity of the 14.5-nm axial reflection was determined by integrating from reciprocal spacings of 1/13.4 to 1/15.8 nm⁻¹ axially and 1/150 nm⁻¹ on either side of the meridian. The background under the reflection was determined by polynomial fitting and subtracted. The remaining reflection intensity corresponded to about 100 counts per 0.2 ms before the step in the fibre in Fig. 1a. Other integration limits gave similar time courses and relative amplitudes but poorer signal to noise ratios. Consistent with this observation, and with previous results at 1 ms time resolution from whole muscles¹³, there was no change in the shape or position of the reflection following the length step, either in its axial width, width perpendicular to the axis, or axial spacing. Although changes in these parameters could not be measured as precisely as those in total intensity, we estimate that a width increase of more than about 10% of the pre-step value with a similar time course to the intensity change can be excluded. The absence of such width increases rules out various types of disorder as possible origins of the intensity change¹³. The lack of an intensity change during the length step itself excludes possible length- or strain-dependent artefactual origins of the change in 14.5-nm reflection intensity.

stroke can be regenerated at a rate of about 100 s⁻¹, which is 10 times higher than the ATP turnover rate per myosin head during steady shortening under otherwise comparable conditions^{12,23}. This behaviour implies several elementary working strokes per ATP molecule hydrolysed¹⁷.

To provide a quantitative test of the main features of conventional models for force generation we combined a simple structural model for the working stroke (Fig. 2) with a detailed mechanical kinetic scheme^{24,25}. Myosin heads of fixed axial width *a* are considered to be bound to the actin filament and connected to the myosin filament by an elastic element. The axial repeat of the heads, *L*, is 14.5 nm. Force is generated by heads tilting while attached to actin to stretch the elastic element by *s*. This can spread out the mass of the heads along the filament, decreasing the intensity of the 14.5-nm X-ray reflection^{13,26}. In the mechanical kinetic scheme there are three states (*A*₁, *A*₂ and *A*₃) in which the myosin head is attached to actin. The transitions *A*₁ to *A*₂ and *A*₂ to *A*₃ each generate force by stretching the elastic element by 4.5 nm, a value which had been chosen to explain the mechanical properties of muscle, including the response to shortening steps^{24,25}. The rates of transitions between these states and of attachment and detachment depend on the extension of the elastic element^{5,9} and had also been chosen to fit mechanical data. The predicted time courses of the fraction of myosin heads in states *A*₁, *A*₂ and *A*₃ after a 6-nm shortening step are shown in Fig. 3.

The predicted change in the intensity of the 14.5 nm reflection was calculated from the *A*₁, *A*₂ and *A*₃ time courses using the tilting head model for the working stroke (Fig. 2). The predicted change (Fig. 3) is very sensitive to the axial shift between the states. A satisfactory fit to the experimental X-ray data was obtained with a value of 4.5 nm for this parameter, in agreement with the extension of the elastic element required to fit

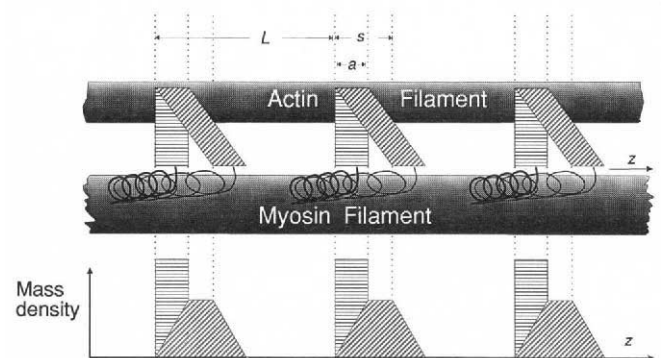


FIG. 2 Simple structural model for the working stroke. Top panel, rectangular myosin head of axial extent *a* tilting to produce an axial displacement *s* of one end of the head with respect to its original position and stretching the elastic element by a corresponding amount. Lower panel, resulting axial mass distribution, where *a* can be generalized to represent the axial extent of a rectangular distribution of myosin heads, which has been assumed to be constant. These distributions occur with axial periodicity 14.5 nm (*L*). The particular myosin head orientations shown here are not considered to represent those in contracting muscle (see Fig. 3). In general we consider three populations of myosin heads (*A*₁, *A*₂, *A*₃) with fractional occupancies *f*₁, *f*₂, *f*₃ (Fig. 3) and *s* values *s*₁, *s*₂, *s*₃. The fractional change in diffracted intensity, obtained from the Fourier transform of density distribution²⁶, is:

$$\begin{aligned} & \frac{f_1^2}{s_1^2} \sin^2(\pi s_1/L) + \frac{f_2^2}{s_2^2} \sin^2(\pi s_2/L) + \frac{f_3^2}{s_3^2} \sin^2(\pi s_3/L) \\ & + 2 \frac{f_1 \cdot f_2}{s_1 \cdot s_2} \sin(\pi s_1/L) \sin(\pi s_2/L) \cos(\pi(s_1 - s_2)/L) \\ & + 2 \frac{f_1 \cdot f_3}{s_1 \cdot s_3} \sin(\pi s_1/L) \sin(\pi s_3/L) \cos(\pi(s_1 - s_3)/L) \\ & + 2 \frac{f_2 \cdot f_3}{s_2 \cdot s_3} \sin(\pi s_2/L) \sin(\pi s_3/L) \cos(\pi(s_2 - s_3)/L) \end{aligned}$$

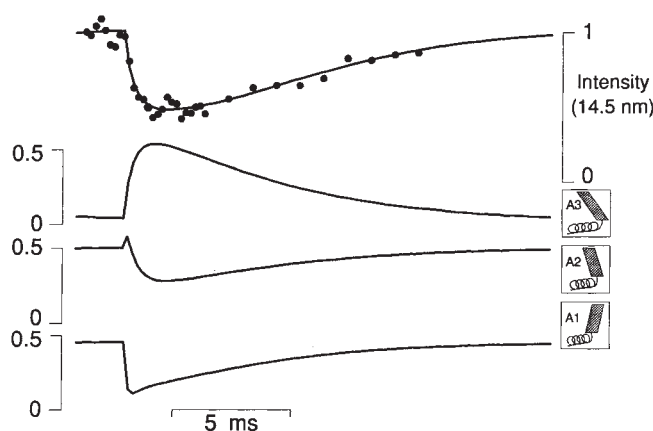


FIG. 3 Changes in intensity of the 14.5-nm axial X-ray reflection ($I(14.5)$; ●) after 6-nm shortening steps. The superimposed continuous line shows predictions from the structural model in Fig. 2 combined with a mechanical kinetic scheme²⁴. X-ray intensity values, at 0.2- or 1.0-ms intervals, are from Fig. 1c. The lower panels show the fractional occupancies (f_1 , f_2 and f_3) of states A_1 , A_2 and A_3 in the mechanical kinetic scheme, calculated as the average values in a series of 10 steps at 20-ms intervals for comparison with the experimental data. The elementary force-generating process is due to the transitions A_1 to A_2 and A_2 to A_3 , each of which stretches an elastic element by 4.5 nm. This kinetic scheme has been described in detail²⁴; minor modifications in a later version²⁵ have also been incorporated here. The predicted intensity of the 14.5-nm reflection is calculated from f_1 , f_2 and f_3 using the model in Fig. 2 with $s_1 = -1.5$ nm; $s_2 = +3$ nm; $s_3 = +7.5$ nm; the angle of tilt of the myosin heads is exaggerated here for clarity. The small fraction of detached states was neglected in calculating the X-ray intensity and the values of f represent the fraction of attached heads. The axial width of the distribution of heads (a in Fig. 2) was assumed to be constant in these calculations. In fact small changes in a are expected after a length step²⁵; further calculations including these terms showed that they have a negligible effect on the predicted intensity.

mechanical data. The present X-ray data do not allow a precise estimate of the offset of the set of three states from the perpendicular orientation, but the s values -1.5 , $+3.0$ and $+7.5$ nm for states A_1 , A_2 and A_3 , respectively, used for the fit in Fig. 3 account satisfactorily for both the amplitude and complete time course of the intensity of the 14.5-nm reflection.

Other structural models for the working stroke could also account for the present data. For example, decreases in the 14.5-nm intensity of similar magnitude could be produced by relative sliding between head populations without tilting. Step shortening produced no significant change in the total integrated intensity of the inner equatorial reflections, which are sensitive to mass transfer in the plane perpendicular to the filament axis, suggesting that mass transfer in the working stroke is predominantly axial. The structural representation of the working stroke in Fig. 2 also oversimplifies the shape of the myosin head, and data from other parts of the diffraction pattern will be required to test more realistic structural models. But the large amplitude of the intensity change requires that a large fraction of the myosin heads move axially by about 10 nm in the elementary force-generating process, independent of the details of the structural or mechanical model. □

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Regulation of vertebrate left-right asymmetries by extracellular matrix

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THE vertebrate body is organized along three geometric axes: anterior–posterior, dorsal–ventral and left–right. Left–right axis formation, displayed in heart and gut development, is the least understood, even though it has been studied for many years^{1–4}. In *Xenopus laevis* gastrulae, a fibronectin-rich extracellular matrix is deposited on the basal surface of ectoderm cells^{5,6} over which cardiac and visceral primordia move during development. Here I report experiments in which localized perturbation of a small patch of extracellular matrix by microsurgery was correlated with localized randomization of left–right asymmetries. Global perturbation of the extracellular matrix by microinjection of Arg–Gly–Asp peptides or heparinase into the blastocoel resulted in global randomization of left–right asymmetries. From these observations, I suggest that left–right axial information is contained in the extracellular matrix early in development and is independently transmitted to cardiac and visceral primordia.

Transplantation, extirpation and wounding experiments on amphibian gastrulae result in reversal of asymmetries along the left–right axis, situs inversus^{7–10}. Surgical wounding of animal pole ectoderm in *Xenopus* early gastrula (stage 10) or explantation and reimplantation of ectoderm in any orientation resulted in reversal rates never exceeding 50% for each of the asymmetric organs. For example, removal and reimplantation of ectoderm in the original orientation and reimplantation after 180° rotation resulted in cardiac situs inversus in 23 out of 50 embryos and 29 out of 60, respectively. The observations in this and previous studies of reversal frequencies of about 50% for each organ suggests that the treatments ‘randomize’ left–right axial information.

The period between early gastrula manipulations that affect left–right development and the morphological expression of left–right asymmetry is the most active period of tissue reorganization in embryogenesis. Consequently, the spatial relationship between the site of manipulation and subsequent left–right reversals of one or more organs was unclear. To assess whether ectodermal wounding is correlated with the randomiz-

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ation of the left-right orientation of the organs that come into contact with the ectoderm during the neurula stages^{11,12}, the site of ectoderm wounding was precisely fate-mapped (Fig. 1). In cases in which the transplanted ectoderm did not directly appose an organ, the left-right orientation of that organ was normal. By contrast, when the transplanted ectoderm directly apposed an organ, the left-right orientation was randomized. Normal and reversed adjacent organs were frequently seen in the same experimental animal (heterotaxia) (Fig. 2). These results indicate that local perturbation of animal pole ectoderm early in gastrulation induces localized randomization of the left-right asymmetry in underlying organs without altering the orientation of nearby organs. This suggests that left-right axial information resides in the ectoderm during gastrulation and is transferred to the cardiac mesoderm and viscera. This is a surprising possibility, because previously described signals for axis formation in early development are thought to pass from mesoderm to ectoderm, not the reverse.

Coincident with the period during which wounding of the ectoderm leads to situs inversus, an extracellular matrix of

fibronectin^{5,6} and heparin sulphate proteoglycans^{13,14} is deposited on the basal surface of the ectoderm. Laminin is not deposited until later¹⁵. In other systems, wounding of epithelial cells leads to reorganization of the extracellular matrix (for review see ref. 16). Less fibronectin was deposited on the small patches of transplanted ectoderm than on any of the controls, including the adjacent, unmanipulated ectoderm (Table 1a). Thus, the correlation of wounding or transplantation of ectoderm and localized randomization of left-right asymmetries could be due to local disruption of normal extracellular matrix deposition.

To perturb the deposition of extracellular matrix experimentally with minimal direct perturbation of the ectoderm, either polypeptides containing the amino-acid sequence Arg-Gly-Asp (RGD)¹⁷ or heparinase were microinjected into the blastocoel. In *Xenopus* gastrulae, the appearance of fibronectin fibrils on the entire blastocoel roof was effectively eliminated by RGD and altered by heparinase (Fig. 3, and Table 1b). In contrast to studies in urodeles¹⁸, RGD treatment did not inhibit gastrulation or the formation of normal anterior-posterior or dorsal-ventral asymmetries. Formation of normal-sized hearts suggest that RGD and heparinase treatment did not noticeably

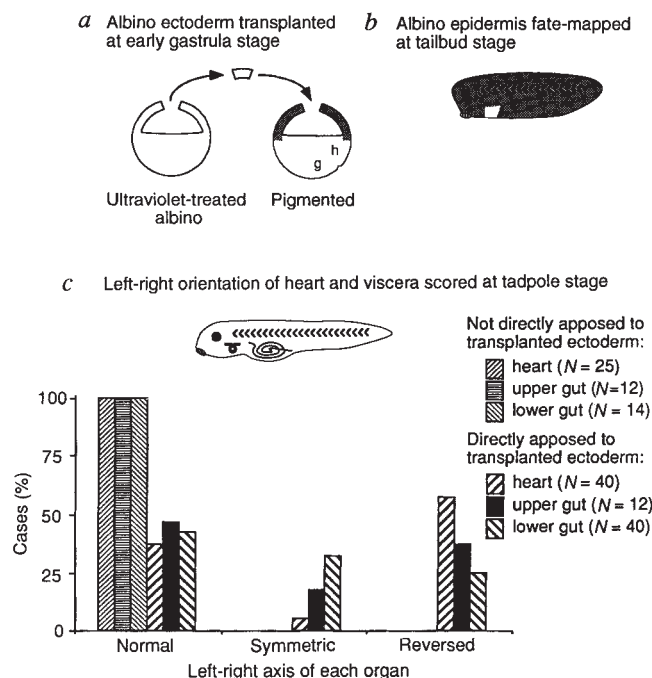


FIG. 1 Local perturbation of animal pole ectoderm during gastrulation results in local randomization of the left-right asymmetry. *a*, In *Xenopus laevis* gastrulae (stage 10), patches of pigmented animal pole ectoderm (roughly 100–150 μm each side) were excised and replaced with transplanted albino ectoderm. To eliminate any possible dorsal-ventral or anterior-posterior bias in the alignment of the transplanted ectoderm, the donor albino embryos had been ultraviolet-irradiated during the first cell cycle at the vegetal pole to inhibit dorsal-ventral and anterior-posterior axis formation²⁴. The transplanted patches healed into the host ectoderm within 30 min. The location of presumptive heart mesoderm (h) and gut endoderm (g) are indicated¹². *b*, The location of the transplanted albino ectoderm was scored in each host embryo at the tailbud stage (stage 28). At this stage, the pigmentation derived from the animal hemisphere of the egg is still present throughout ectoderm of the embryo and no morphological left-right asymmetries are evident. *c*, At stage 45, the egg-derived pigmentation is dissipated and the periodic albinism of the donor ectoderm is not visible. For each tadpole, the left-right orientation of the heart, 'upper gut' (liver, gall bladder and pancreas) and 'lower gut' (placement and handedness of intestinal coil) was scored independently and the results were combined with the ectoderm fate mapping described above. It is unlikely that left-right randomizations were due to either the ultraviolet treatment or the albinism of the donor, because transplanting normal, pigmented animal caps resulted in similar frequencies of reversals (see text). Operations performed in blastocoelic medium²⁵ or a variety of other media had similar results, suggesting that the left-right reversals were not merely a result of altering the blastocoel fluid.

TABLE 1 Immunofluorescent quantification of fibronectin deposited on the basal surface of animal pole ectoderm under various experimental treatments

(a) Surgical wounding and transplantation

Row	Ectoderm	N	Fibronectin (% host)
1	Stage 9 transplant	6	65*
2	Stage 10 transplant	6	37†
3	Stage 10.5 transplant	6	30†
4	Stage 11 transplant	4	64*
5	Peripheral to transplants, stages 9–11	44	100
6	UV-treated albino, not transplanted	10	117
7	Control pigmented, not transplanted	5	98

(b) Injection of solutions

Treatment	N	Fibronectin fluorescence (% control)
Control	10	25.9 $\pm$ 5.8 (100)
Buffer	12	29.2 $\pm$ 6.2 (113)
RGE	9	25.6 $\pm$ 6.9 (99)
Heparinase	15	19.8 $\pm$ 7.4 (76)‡
RGD	23	3.5 $\pm$ 1.4 (13)§

a, Surgical wounding and transplantation resulted in local diminution of fibronectin immunofluorescence. A small rectangle of animal pole ectoderm was transplanted into host embryos at the stages indicated. The donor embryos had been injected with rhodamine-dextran-lysine to identify the transplanted tissue. For each animal cap (N), the ratio of the fluorescence in the transplanted region to the mean fluorescence in the peripheral host regions was calculated. The mean of this ratio for each group of animal caps is shown as per cent host fluorescence in rows 1–4. Controls were host peripheral ectoderm (row 5), ectoderm that had not been surgically manipulated, from either ultraviolet-treated albino embryos (row 6) or control, pigmented, rhodamine-dextran-lysine-labelled embryos (row 7). *b*, The indicated solutions were injected into the blastocoels of stage-10 embryos. The mean intensity of immunofluorescence and standard deviation are shown with the percentage of control group intensity. The immunofluorescence seen in RGD-treated samples was equivalent to background staining (see Fig. 3e). Animal caps were processed as for Fig. 3. Intensity of fibronectin immunofluorescence in a rectangular region (16,727 μm^2) was assessed by confocal microscopy and computer image analysis (Biorad). For each animal cap (N), five scanned images at each of three consecutive 1.0- μm confocal sections were collected and summed. For *a*, rows 1–5, one region in donor ectoderm and two randomly selected regions in adjacent host ectoderm were analysed. *a*, Rows 6 and 7, and *b*, three randomly selected non-overlapping regions were analysed. Significance was determined using Student's *t*-test²⁸.

* $P < 0.03$; † $P < 0.01$; ‡ $P < 0.02$; § $P < 0.0005$.

alter the migration of cardiac primordia to the ventral midline, in contrast to the effects of RGD in chick¹⁹. The instructive exception to the otherwise normal appearance of RGD or heparinase-injected embryos was that the left-right asymmetries of heart, upper gut and lower gut were 'randomized' (Fig. 3). The left-right orientation of each of these three regions was statistically independent of the orientation of neighbouring regions (heterotaxia). The most effective period for either RGD or heparinase treatment was from late blastula to early gastrula (stages 8–10), coincident with fibronectin-rich extracellular matrix deposition. These results suggest that global perturbation of this deposition leads to global randomization of left-right asymmetry. The fact that the orientation of all organs normally appears to be linked perhaps results from a mechanism by which each organ primordia independently acquires left-right axial information from a unified source. The only genetic model of situs inversus, the *iv/iv* mutant mouse, also displays heterotaxia (for review, see ref. 2). It will be interesting to learn whether the product of the *iv* locus, which has been mapped²⁰, is involved in an extracellular matrix interaction pathway.

To extend the theoretical model by Brown and Wolpert², we can now distinguish experimentally three developmental stages

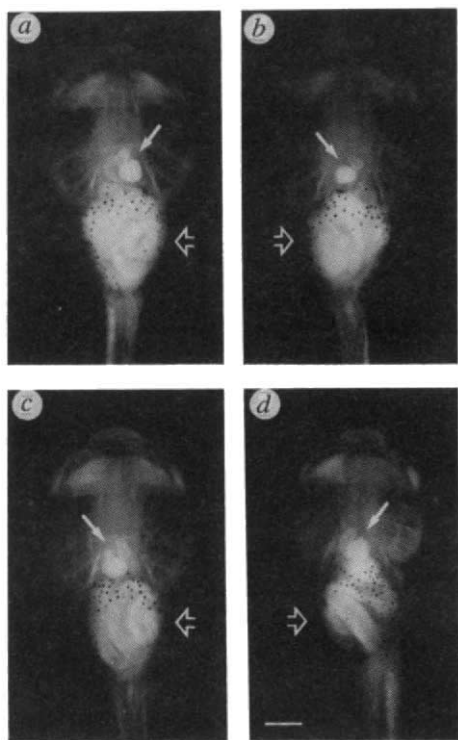


FIG. 2 Ventral view of stage 45 tadpoles from ectoderm transplantation experiments described in Fig. 1. Anterior is towards the top, the heart is indicated by closed arrow, intestinal coil is indicated by open arrow. Scale bar, 0.5 mm. **a**, Tadpole displaying normal left-right asymmetry formation. Tracing the heart from anterior to posterior, the conus, ventricle and atrium (below the photographed focal plane) form the 'S' shape seen in all vertebrates. The intestinal coil is counterclockwise, with the shield of the coil on the tadpole's left. In this tadpole, the transplanted ectoderm directly apposed the heart and upper gut, but not the lower gut. **b**, Situs inversus totalis. In this tadpole, the transplanted ectoderm directly apposed the heart, upper gut and lower gut, and the left-right asymmetry was completely reversed in all these organs. The heart was reversed 'S' shape, the liver, gall bladder and pancreas were in reversed orientation and on the left side, and the intestine coil was clockwise and on the right side. **c**, Tadpole in which the heart was situs inversus and the gut was normal. The transplanted ectoderm directly apposed the heart and upper gut, but not the lower gut. **d**, Tadpole with heart, liver, gall bladder and pancreas in normal orientation, and lower gut situs inversus. The transplanted ectoderm directly apposed only the lower gut.

of left-right axis formation. First, the left-right axis is established globally, in coordination with the dorsoventral and anteroposterior axes. Experimental manipulation of *Xenopus* one-cell embryos²¹ or of *Caenorhabditis elegans* four-cell embryos³ results in an 'all-or-none' reversal of the left-right asymmetry of all organs (situs inversus totalis). Second, my results indicate that left-right axial information is subsequently dependent on and possibly localized in ectodermal extracellular matrix in the gastrula. Each organ primordium, as it contacts the matrix in a localized fashion, seems independently to interpret the left-right axial information. The transitional events that occur between the initial establishment of the left-right axis and placement of left-right information in the gastrula are not known. Third, the expression of this left-right information occurs later in development as a morphological conversion of bilateral symmetry to asymmetry, which can be experimentally inhibited¹³.

Extracellular matrix asymmetry interpreted by the underlying heart and gut primordia could be in two forms. First, a global alignment of fibrils that have an intrinsic molecular handedness would present an asymmetric surface to the primordia. In *Xenopus*, although the extracellular matrix is necessary for directional migration of prospective head and axial mesoderm cells²², the alignment of fibronectin fibrils appears to be random both at the beginning of gastrulation⁶ and near the end of gastrulation

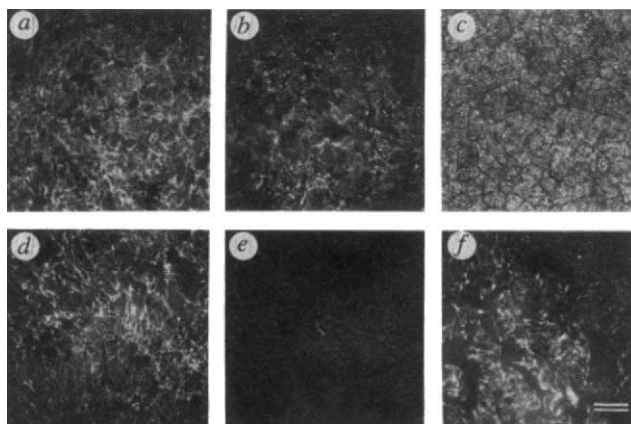


FIG. 3 Immunofluorescence micrographs of fibronectin fibrils on the basal surface of animal pole ectoderm from late gastrula (stage 12). Microinjections were performed at the beginning of gastrulation (stage 10). Scale bar, 0.05 mm. **a**, Uninjected gastrula. Large fibrillar network spans several cell diameters (see **c**). **b**, Gastrula injected with buffer as a control. **c**, Same as **b**, except transmitted luminescence shows outlines and basal surfaces of ectodermal cells. **d**, Gastrula injected with RGE as a control. **e**, Gastrula injected with RGD. The fibronectin fibril shown in the centre of this photograph was the only fibril found among eight independent RGD-treated preparations. **f**, Gastrula injected with heparinase. Left-right asymmetries were randomized in a parallel series of embryos injected with either RGD (46% situs inversus, $N=37$) or heparinase (40% situs inversus, $N=20$). In this and other experiments, situs inversus did not occur in uninjected embryos ($N=381$) or embryos injected with buffer ($N=272$) or heat-inactivated heparinase ($N=87$), and only rarely (7 of 189) in embryos injected with RGE. Whether the randomizations observed with heparinase treatment are due to a direct effect of the enzyme on heparin sulphate proteoglycans or an indirect result of the decrease in fibronectin deposition (Table 1b) is unclear. Microinjection of stage 10 embryos with smaller doses of RGD (6.5 mg ml^{-1}) resulted in 20% situs inversus ($N=59$). RGD or heparinase injection at a time after the fibronectin-rich ECM had been deposited (late gastrula, stage 12) had little or no effect on left-right development. **METHODS.** Embryos were staged according to Nieuwkoop and Faber²⁶. Final concentration of peptides (GRGDSP, GRGDTP and GRGESP; Telios) was about 13 mg ml^{-1} and heparinase (Sigma) was about 100 unit ml^{-1} . Embryos were fixed at stage 12, animal caps were removed and processed for immunohistochemistry²⁷ with anti-fibronectin polyclonal antibody (Calbiochem) and fluorescent secondary antibody.

(Fig. 3). But, during the neurula stages, coincident with movement of the cardiac and gut primordia across the extracellular matrix, the ectoderm is stretched by elongation of the embryo in the anterior-posterior axis¹¹. Stretching the ectodermal sheet to which the fibrils are attached could align the fibrils parallel to the axis of elongation. Second, there could be quantitative or qualitative differences from left to right either in extracellular matrix components or factors anchored to it which are eliminated by a perturbation of normal matrix deposition. In other systems, a number of growth factors are known to be sequestered in the extracellular matrix²³. □

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Profound block in thymocyte development in mice lacking p56^{lck}

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THE protein Lck (p56^{lck}) has a relative molecular mass of 56,000 and belongs to the Src family of tyrosine kinases¹⁻³. It is expressed exclusively in lymphoid cells, predominantly in thymocytes and peripheral T cells^{4,5}. Lck associates specifically with the cytoplasmic domains of both CD4 and CD8 T-cell surface glycoproteins^{6,7} and interacts with the β -chain of the interleukin-2 receptor⁸, which implicates Lck activity in signal transduction during thymocyte ontogeny⁹⁻¹³ and activation of mature T cells¹⁴⁻¹⁹. Here we generate an *lck* null mutation by homologous recombination in embryonic stem cells to evaluate the role of p56^{lck} in T-cell development and activation. Lck-deficient mice show a pronounced thymic atrophy, with a dramatic reduction in the double-positive (CD4⁺CD8⁺) thymocyte population. Mature, single-positive thymocytes are not detectable in these mice and there are only very few peripheral T cells. These results illustrate the crucial role of this T-cell-specific tyrosine kinase in the thymocyte development.

For homologous recombination, a replacement-type vector (PmlckBSNeo2.3) was constructed (Fig. 1a) and introduced into D3 embryonic stem cells by electroporation. Six independently targeted embryonic stem cell lines were generated. The average frequency of homologous recombination was about 1 in 2×10^7 electroporated cells and 1 in 130 G418-resistant clones. Southern blot analysis confirmed the homologous recombina-

tion event (Fig. 1b). Germ-line transmission of the *lck* disruption was obtained with two embryonic stem cell lines. Heterozygous mice were inbred to obtain mice homozygous for the disrupted *lck* gene.

Although mice homozygous for the disrupted *lck* gene are expected to lack p56^{lck}, it was conceivable that part of the Lck protein to the amino-terminal side of the mutation could still have been expressed. Therefore for western blotting we used a rabbit antiserum directed against the N-terminal end of Lck, using either thymic (Fig. 2a) or lymph node cell lysates (data not shown). No Lck protein, either full-length or truncated, was detected in homozygous mutant mice. In addition, immune complex kinase was assayed using a different rabbit anti-Lck antiserum and there was no detectable p56^{lck} kinase activity in homozygous mutant mice (Fig. 2b).

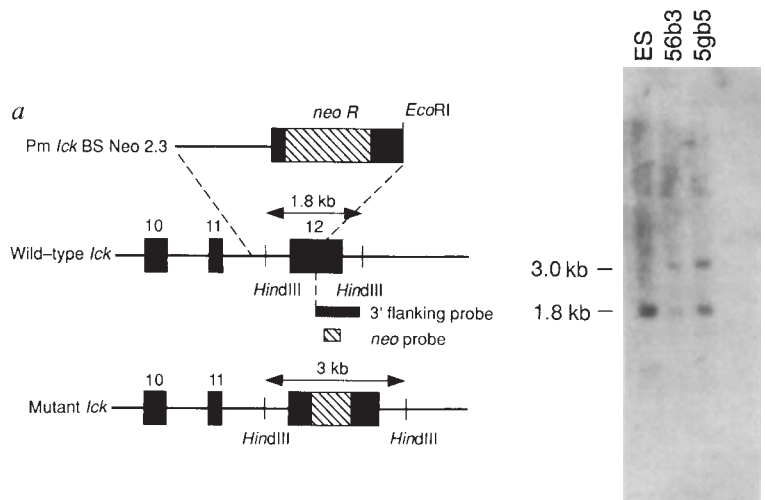
Heterozygous mutant mice appeared healthy; their lymphoid organs and thymocyte and peripheral T cell populations were normal (data not shown). But CD4 staining of peripheral blood lymphocytes showed a consistent decrease in fluorescence intensity (Fig. 3a); this decrease was not seen on CD4⁺ thymocytes, nor was there any downregulation of CD8 expression (data not shown).

Lck-deficient mice appeared healthy and fertile under specific pathogen-free conditions. Spleen and lymph nodes were normal in size but we observed considerable thymic atrophy; conventional histology showed a thymic cortex sparsely populated with lymphoid cells and a very tiny medulla (data not shown). Thus thymic atrophy is associated with a decrease in the thymocyte population (6×10^6 – 2×10^7 cells versus 2×10^8 for normal littermates), consisting of 20–40% double negative (CD4[−]CD8[−]) and 60–80% double positive (CD4⁺CD8⁺) thymocytes. The number of double negative thymocytes is similar to that of normal littermates (3×10^6 – 6×10^6), but there is a dramatic reduction in double-positive thymocytes (0.3×10^7 – 1.6×10^7 versus 1.8×10^8 for normal littermates), evident in mice from 10 days to 8 weeks old. Neither CD4⁺CD8[−] nor CD4[−]CD8⁺ single-positive thymocytes were detectable in homozygous mutant mice (Fig. 3b).

The double-negative thymocyte population is Thy-1⁺ CD3 (low-intermediate), with the presence of a J11d (high), interleukin-2(IL-2) receptor α -chain-positive subpopulation (40% of double-negative thymocytes). The double-positive thymocyte population does not contain CD3 (low) cells and there is, on average, increased CD3 expression at intermediate to high levels: 25% of the CD3⁺ cells have an expression level similar to that of normal CD3 (high), single-positive thymocytes

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FIG. 1 Disruption of *lck* gene by homologous recombination. **a**, Schematic diagrams of the targeting vector (PmickBSNeo2.3), the *lck* locus in parental D3 cells (wild-type *lck*), and the predicted structure of the disrupted *lck* locus (mutant *lck*). The neomycin-resistance gene (*neo R*) cassette contains 1.2 kilobases (kb) derived from the *XhoI*-*SalI* fragment of plasmid pMC1PoA (ref. 28) and was inserted into a *SalI* site created at a previous *Bsp*MI site in exon 12 of the *lck* gene, 110 base pairs (bp) upstream of the codon corresponding to Tyr 505. The 2.3 kb of genomic *lck* in the targeting vector was obtained by screening of a mouse (BALB/c) genomic library with a human *lck* complementary DNA probe². Electroporation of the D3 embryonic stem cells and screening for homologous recombination were done as previously described^{25,26}. A primer specific for the *neo* cassette in the neomycin-resistance coding sequence (5'-TATCAGGACATAGCGTTGGCTACCC-3'), and another antisense primer specific for exon 12 of *lck*, 3' of the targeting vector (5'-CTTAGACTCAGTGCTCCACAGTA-3'), were used in the polymerase chain reaction (PCR) to detect homologous recombination; *lck* cDNA sequences spanning exon 12 were used as a probe in this PCR detection method (data not shown). **b**, Southern blot analysis of the structure of the *lck* locus in parental D3 cells (ES) and two targeted cell lines (56b3 and 5gb5). Genomic DNA was digested with *Hind*III and probed using the 3' flanking probe (600 bp *Eco*RI-*Hind*III fragment). In cases of homologous recombination, a 3-kb fragment is



(Fig. 3b). The drastic reduction of double-positive thymocytes may reflect an inability of this cell population to enter the cell cycle. However, propidium iodide staining²⁰ of thymocytes from 4-week-old homozygous mutant mice showed a cell-cycle profile similar to that of normal littermates (+/+; ref. 21): about 20% of thymocytes were in the S or G2+M phase of the cell cycle (data not shown).

Although the overall numbers of cells in lymph nodes and spleens are normal, a dramatic inversion in the B/T cell ratio

detected from the disrupted *lck* allele, instead of a 1.8-kb fragment from the wild-type allele. The same profile of hybridization was observed using the targeting vector as a probe, whereas probing with the neomycin-resistance gene showed only the single 3-kb fragment (data not shown).

was observed: 90 to 95% B cells and 5 to 10% T cells (Fig. 3c). Peripheral T cells are positive for Thy-1, CD3 and for the α - and β -chains of the T-cell antigen receptor (TCR $\alpha\beta$ ⁺) and expression of CD4 or CD8 (Lyt2-Lyt3 heterodimer; Fig. 3d) is decreased. One per cent of the lymph node population are TCR $\gamma\delta$ ⁺ T cells (data not shown).

Proliferative responses of peripheral T cells to mitogenic stimuli were identical for both normal and heterozygous mutant mice (data not shown). T cells from *Lck*-deficient mice exhibited

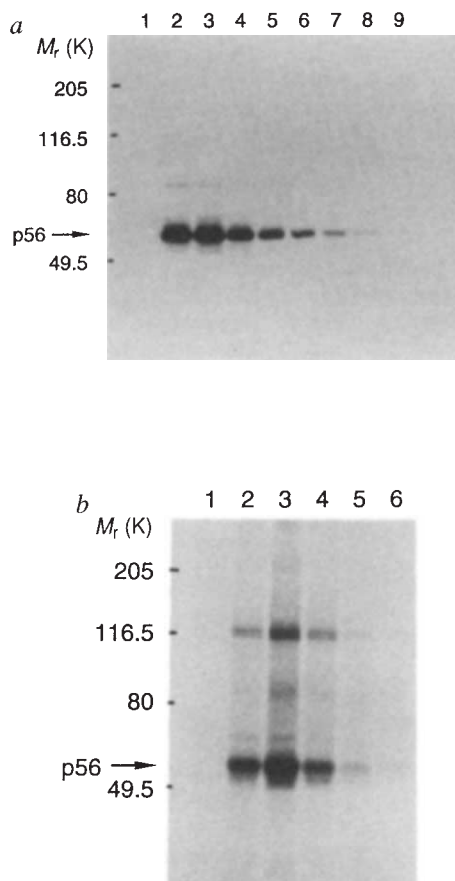


FIG. 2 Analysis of p56^{lck} expression and activity. **a**, Immunoblot quantitation of p56^{lck} protein. Thymocyte lysates (100 μ g) are shown from homozygous mutant (-/-; lane 1), heterozygous mutant (+/-; lane 2), and wild-type mice (+/+; lane 3); lanes 4-9, titration of wild-type (+/+) thymocyte lysate (50, 25, 12.5, 6.25, 3.12 and 1.6 μ g, respectively). Autoradiograph exposure time was 15 h. Quantitation of the bands revealed that the amount of p56^{lck} in the heterozygous mutant mouse cell lysate (lane 2) was roughly half of the amount in wild-type mouse lysate (lane 3). **b**, Immune complex kinase assay of p56^{lck} activity. The tyrosine kinase activity of p56^{lck} (autophosphorylation) was assessed using *Lck* immunoprecipitates from cell lysates: 150 μ g thymocyte lysates from homozygous mutant (-/-; lane 1), heterozygous mutant (+/-; lane 2), and wild-type mice (+/+; lane 3); lanes 4-6, titration of the wild-type (+/+) thymocyte lysate (75, 37.5, and 18.8 μ g, respectively). Autoradiograph exposure time was 15 h. Quantitation revealed that the kinase activity in the heterozygous mutant mouse cell lysate (lane 2) was roughly half of the wild-type activity (lane 3). Positions of markers of known molecular weight and the position of p56^{lck} are indicated on the left of each figure.

METHODS. Immunoblot analysis: Thymic and lymph node cells of 3-4-week-old mice were washed in PBS and lysed in TNE buffer (50 mM Tris, pH 8.0, 1% NP40, 2 mM EDTA) supplemented with protease inhibitors and phosphatase inhibitors as before¹⁴. Defined amounts of proteins from cellular lysates were denatured in sample buffer, boiled and resolved by 8% SDS-PAGE. Proteins were electrophoretically transferred to nitrocellulose and analysed for p56^{lck} expression using a rabbit antiserum generated against a fusion protein containing amino acids 2-148 of the murine p56^{lck} sequence¹⁶. Bands were cut from the nitrocellulose blot and counted in a γ -counter. Immune complex kinase assay: p56^{lck} was recovered from cell lysates using a rabbit anti-*Lck* antiserum directed against amino acids 39-64. After collection on formalin-fixed *Staphylococcus aureus* (Pansorbin, CalBiochem), immune complexes were washed and resuspended in kinase buffer¹⁴. Kinase reactions were performed for 2 min at room temperature with constant shaking in 25 μ l kinase buffer containing 1 μ M in labelled ATP, 12.5 μ M $[\gamma$ -³²P]-ATP (3,000 Ci mmol⁻¹; NEN). Reactions were stopped by addition of 1 ml lysis buffer, washed three times, and resuspended in sample buffer. Phosphorylated polypeptides were subsequently resolved on 8% SDS-PAGE gels.

a proliferative response, albeit reduced, to both CD3 and TCR $\alpha\beta$ crosslinking (Fig. 4a), demonstrating that Lck is not indispensable for the TCR-CD3 signalling pathway and may act as a signal amplifier¹⁶. In addition, the enhanced response to CD3 or TCR crosslinking in the presence of IL-2, as well as the response to IL-2 alone (Fig. 4a), suggests that p56^{lck} is not indispensable for the IL-2 signalling pathway⁸. The responses to phorbol ester and ionomycin treatment were identical in normal and Lck-deficient T cells.

Evaluation of serum immunoglobulin levels revealed an increase in IgM, a significant decrease in IgG₁, and a marginal increase in IgG_{2a} and IgG₃ isotypes in homozygous mutant mice (Fig. 4b), representing an immunoglobulin profile comparable to that seen in the absence of T-helper cell function (for example, major histocompatibility complex class II-deficient mice²² and IL-4-deficient mice²³). Splenic B cells responded normally to lipopolysaccharide (Fig. 4b), however, demonstrating that the

lack of *lck* does not interfere with T-independent B-cell function.

The profound perturbation of thymocyte development in Lck-deficient mice underlines the crucial role of this kinase in T-cell ontogeny, because the other T-cell-specific tyrosine kinases, p59^{fyn}^T and p62^{yes}, are unable to compensate for the absence of p56^{lck}. This lack of functional redundancy is surprising in light of the paucity of abnormalities present in Src-deficient mice²⁴ and the observation of normal thymocyte development and peripheral immune responses in mice lacking *fyn* (P. Stein and P. Soriano, personal communication).

The interaction between p56^{lck} and the accessory molecules CD4 and CD8 in double-positive thymocytes¹¹, as well as the enhancement of Lck activity upon CD4 crosslinking¹⁶, suggest that the impairment of thymocyte maturation in Lck-deficient mice reflects an inability of CD4 and/or CD8 to transduce their signal(s). But both CD4-deficient and CD8-deficient mice have normal thymic architectures and no decrease in the total number

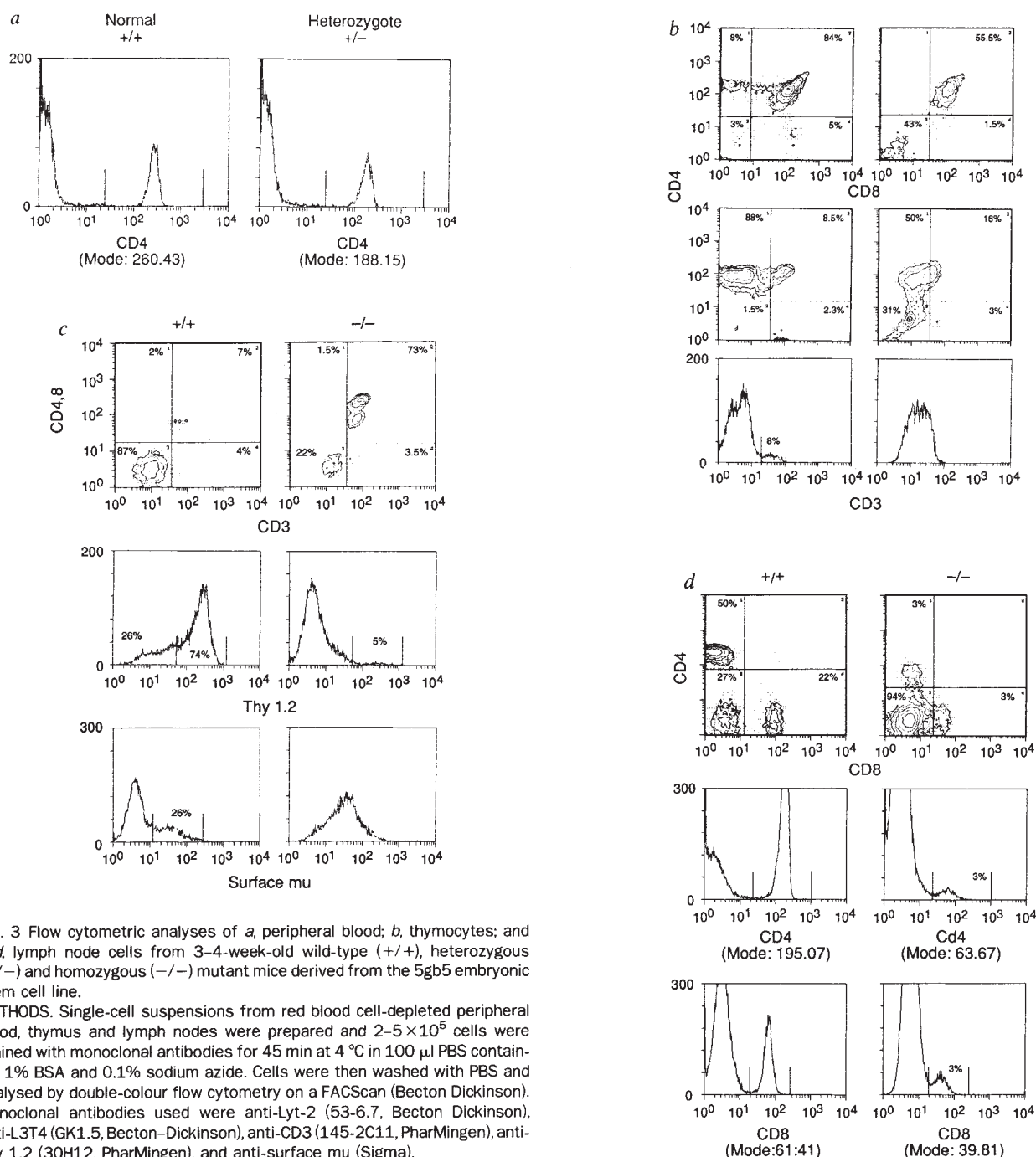


FIG. 3 Flow cytometric analyses of *a*, peripheral blood; *b*, thymocytes; and *c*, *d*, lymph node cells from 3–4-week-old wild-type (+/+), heterozygous (+/-) and homozygous (-/-) mutant mice derived from the 5g5 embryonic stem cell line.

METHODS. Single-cell suspensions from red blood cell-depleted peripheral blood, thymus and lymph nodes were prepared and $2-5 \times 10^5$ cells were stained with monoclonal antibodies for 45 min at 4 °C in 100 μ l PBS containing 1% BSA and 0.1% sodium azide. Cells were then washed with PBS and analysed by double-colour flow cytometry on a FACScan (Becton Dickinson). Monoclonal antibodies used were anti-Lyt-2 (53-6.7, Becton Dickinson), anti-L3T4 (GK1.5, Becton-Dickinson), anti-CD3 (145-2C11, PharMingen), anti-Thy 1.2 (30H12, PharMingen), and anti-surface mu (Sigma).

of thymocytes^{25,26}. Therefore, an inability to signal through CD4 or CD8 cannot be considered as the main factor responsible for the thymocyte developmental defect in Lck-deficient mice.

Whereas CD4- and CD8-mediated signals are considered important during positive selection^{12,13}, the signalling pathway(s) involved in the prior phase of expansion of the double-positive thymocyte population are still unknown²⁷. The drastic

reduction in the number of double-positive thymocytes observed in Lck-deficient mice therefore implicates p56^{lck} in the signalling pathway required for this expansion phase, but does not exclude the possibility that p56^{lck}-mediated signals are also important during thymic selection events. □

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Tetrameric cell-surface MHC class I molecules

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PURIFIED major histocompatibility complex (MHC) class I molecules have been studied at high resolution by X-ray crystallography¹; the structure is a complex of a single heavy chain, a β_2 -microglobulin light chain and a tightly bound peptide moiety. We show here that complete MHC class I molecules are post-translationally assembled into tetramers (made up of four heavy chains and four β_2 -microglobulin units) and that this tetrameric species is expressed on the cell surface. The multivalent tetrameric structure of class I molecules can be reconciled with models of T-cell activation that invoke antigen-receptor crosslinking, as opposed to models that depend on an allosteric change.

Lymphoid cell lines were metabolically labelled and lysed with 1% digitonin with or without a cleavable crosslinking reagent. MHC class I molecules were immunoprecipitated from control and crosslinked lysates and samples analysed on non-reducing, reducing and two-dimensional non-reducing/reducing polyacrylamide-sodium dodecyl sulphate gels. On non-reducing gels, in addition to a free heavy chain band migrating at a position corresponding to an M_r of 45,000 (45K), and a 60K heavy chain- β_2 -microglobulin complex, a species of 240K was seen (Fig. 1, lanes 3 and 5). This band was not observed

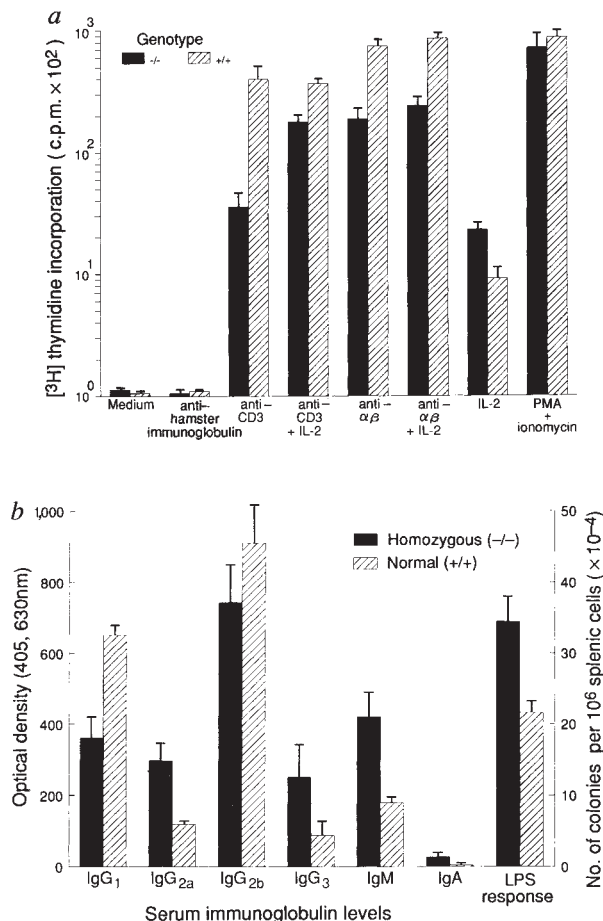
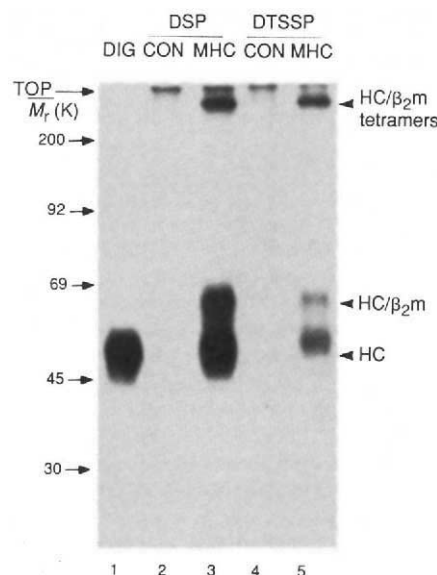


FIG. 4 Analyses of peripheral T-cell and B-cell function. *a*, Proliferative responses of peripheral T cells from normal (+/+) and Lck-deficient (-/-) mice. Values represent mean of triplicate cultures $\pm$ standard deviations. This experiment is representative of four different experiments. *b*, B-cell responses in normal (+/+) and Lck-deficient (-/-) mice. Serum immunoglobulin levels and splenic cell lipopolysaccharide (LPS) responsiveness were analysed by a modified enzyme-linked immunosorbent assay and an agar colony assay, respectively.

METHODS. T cell proliferation: Lymph node and splenic T cells from normal, heterozygous (data not shown), and homozygous mutant mice were purified using nylon wool and cultured in flat-bottomed 96-well plates at a concentration of 5×10^4 T cells per well (as measured by Thy-1 and CD3 staining on FACSscan). For crosslinking experiments plate-bound antibody stimulation was used and 10^6 irradiated (3,000 rads) spleen cells were added. Before stimulation with anti-CD3 (2C11) and anti-TCR $\alpha\beta$ (H57-597) antibodies, plates were coated overnight with purified rabbit anti-hamster IgG ($10 \mu\text{g mL}^{-1}$). Concentrations used were 20 units per ml recombinant interleukin-2 (Roche), 10 ng mL^{-1} phorbol 12-myristyl 13-acetate (PMA; Sigma) and 400 ng mL^{-1} ionomycin (CalBiochem). Cells were cultured for 3 days in IMDM medium with 10% FCS, pulsed with $1 \mu\text{Ci}$ [^3H]thymidine on day 3 and collected 9 h later. B cell responses: The immunoglobulin isotypes in 5-week-old homozygous mutant (-/-) and age/sex-matched normal (+/+) mice were determined by a panel of mouse isotyping antibodies (Bio-Rad), according to the manufacturer's protocol, using sera diluted 100 times in PBS. The frequency of lipopolysaccharide-responsive splenic cells was determined by a double-layer agar culture assay²⁹. Spleens of homozygous mutant mice contained, on average, ~30% more B cells (data not shown). Optical densities are given in arbitrary units.

FIG. 1 Analysis of class I species using chemical crosslinking. 3320-11 cells were metabolically labelled for 18 h using [35 S]methionine and lysed with 1% digitonin with or without (lane 1; DIG, digitonin only) a cleavable chemical crosslinker. The crosslinkers used were dithiobis(succinimidyl)-propionate (DSP; Pierce) (lanes 2 and 3) and dithiobis(sulphosuccinimidyl)-propionate (DTSSP; Pierce) (lanes 4 and 5). Half of each crosslinked sample was immunoprecipitated with anti-class I antibodies (MHC; lanes 3 and 5); the other half was immunoprecipitated with non-immune rabbit IgG (CON; lanes 2 and 4). Samples were analysed on a 9% polyacrylamide-SDS gel. **METHODS.** Cell lines: 3320-11 is an Abelson B cell line (C57/B6 background; S.P., unpublished) which corresponds to the transitional B stage^{6,7}, AJ9 (ref. 8) (A/J) and WEHI 231 (ref. 9) (BALB/c) and D2N (American Type Culture Collection, Bethesda) are B lymphomas. Cells were labelled for 18 h ($\sim 5 \times 10^7$ cells in 40 ml complete medium with 0.5 to 1 mCi of [35 S]methionine), washed in PBS and lysed either in 1 ml of 1% digitonin (WAKO) in PBS (uncrosslinked portion), or in the detergent made 1.25 mM with respect to DSP or 5 mM with respect to DTSSP. In both cases the lysis buffer included freshly added 2 mM phenylmethylsulphonylfluoride. After 30 min on ice, 0.1 ml 50 mM glycine was added to quench unreacted crosslinker and lysates were centrifuged for 5 min at 1,000g to remove nuclei, and then at 14,000g for 20 min at 4 °C. One half of each supernatant was immunoprecipitated with anti-MHC class I antibodies (28-14-8S, anti L^d, D^b (ref. 10); 20-8-4, anti K^d, K^e, D^b (ref. 2)) or with nonimmune rabbit IgG. In some experiments an isotype matched monoclonal antibody was used as a control. Immunoprecipitation was completed with protein A-Sepharose and beads were washed with 0.2% digitonin in 10 mM Tris, pH 7.6, 4 mM EDTA, 150 mM NaCl before analysis on a non-reducing 9% polyacrylamide-sodium dodecyl sulphate gel¹¹. Arrow at the top left corner marks the top of the gel. Positions of molecular weight markers (Amersham) are indicated on the left. The band



labelled HC (heavy chain) corresponded to an M_r of 45K, the band labelled HC/ β_2 m (heavy chain/ β_2 -microglobulin) migrated at 60K, and the tetramer band migrated at a position corresponding to 240K on a linear extension of the semilogarithmic plot.

in the absence of crosslinkers (lane 1) or when irrelevant antibodies were used for immunoprecipitation (lanes 2 and 4). Although in all cases shown here we simultaneously added crosslinker with 1% digitonin as detergent for lysis, the 240K species could also be identified when 1% Nonidet P-40 (NP40) detergent was used instead and when crosslinking preceded lysis (data not shown).

It has been observed² that soon after synthesis, MHC class I heavy chains are transiently associated with an 88K putative chaperone in the endoplasmic reticulum (ER); this 88K protein is an intracellular, presumably ER-specific protein, and can be crosslinked to free heavy chain to yield a 140K species². In these earlier studies the 88K protein was poorly labelled by metabolic labelling; the 140K crosslinked complex was a transient intracellular intermediate and hence the 88K putative chaperone could not be detected by cell-surface iodination, although some of its tyrosine residues are accessible to iodination². As we saw minimal amounts of this 140K crosslinked protein species when

cells were steady-state labelled (for about 18 hours in complete medium), we repeated our experiments with a shorter pulse period followed by crosslinking and lysis at different chase points (Fig. 2a). Crosslinked species seen soon after synthesis include a 60K species, a 140K species and a small amount of the 240K species. On a later 2-h chase point the relative proportion of free heavy chain diminishes, the 140K species is no longer seen and the 240K species is a major product. Complexes after 5 h chase (Fig. 2b) revealed no loss of the 240K species, suggesting that this crosslinked species is not a transient intermediate. Analysis on a two-dimensional gel system (Fig. 3) confirmed that the minor 140K species contained MHC heavy chains and the poorly labelled 88K putative chaperone, whereas the prominent 240K species contained free heavy chains and β_2 -microglobulin and no other detectable metabolically labelled protein species. The size of this species suggested that it represents a tetramer made up of four heavy-chain and four β_2 -microglobulin units.

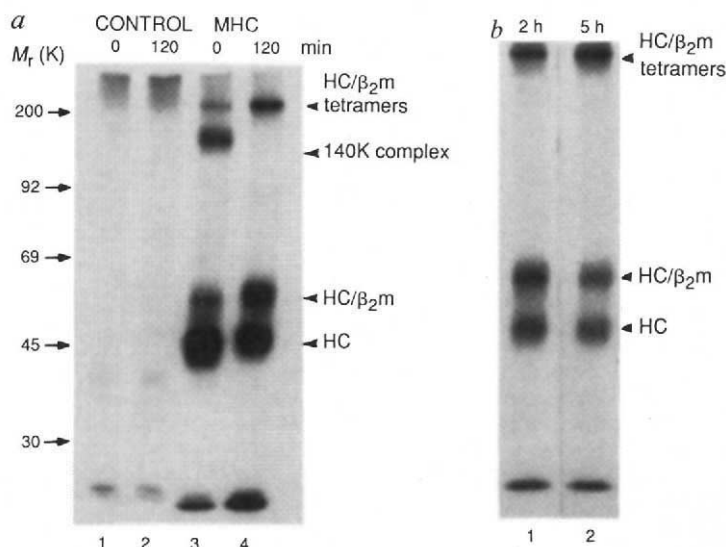


FIG. 2 Pulse chase analysis of crosslinked MHC species. **a**, 3320-11 cells (5×10^7 cells in 1 ml methionine-free medium) were starved for 1 h, labelled for 10 min with 1 mCi [35 S]methionine and one half of the cells were chased in complete medium for 2 h before lysis and crosslinking (120 min; lanes 2 and 4). The other half were lysed and crosslinked immediately after the pulse period (0 min; lanes 1 and 3). The chemical crosslinker was DSP. Anti-MHC antibodies (lanes 3 and 4) were used as described for Fig. 1. An isotype-matched monoclonal antibody 12 CA-5 (ref. 12) was used as a control (lanes 1 and 2). The slight differences in mobility between the two chase points for the free heavy chains have been previously ascribed to the processing of *N*-linked carbohydrates¹³. Other details as for Fig. 1b. In this panel the 2-h and 5-h chase points are demonstrated in an experiment identical to that shown in **a**. After crosslinking and lysis, immunoprecipitation was done using the 28-14-8S antibody alone.

Cell-surface iodination, digitonin lysis and crosslinking revealed the presence of the 240K MHC class I species on the cell surface (Fig. 4). Two-dimensional analysis of immunoprecipitates from crosslinked lysates of surface-iodinated cells revealed no additional proteins other than MHC class I heavy chains and β_2 -microglobulin in the tetrameric species and, as expected, no 140K complex (Fig. 4a). This finding confirms that the 240K species does not involve association with the putative ER chaperone (which is totally intracellular) and that this tetrameric species is transported to the cell surface. When the fate of cell-surface-iodinated tetramers was investigated in a 'chase' experiment (Fig. 4b), tetramers were still detected even after cells had been reincubated in culture medium at 37 °C for 5 hours; the kinetics of disappearance of tetramers were similar to those of uncrosslinked monomers and the residual 60K species. Chemical crosslinking in cell lysates is rarely quantitative; using such an approach it is not possible to state with certainty that all mature class I molecules are tetramers. The results depicted in Figs 2b and 4b suggest, however, that this may well be the case.

In metabolic labelling experiments we either bring down 60 K 'monomers' or 240K tetramers but no 120K dimers or 180K trimers; tetramers are presumably more stable than dimers and trimers. All our results involved 3320-11, a murine Abelson B-cell line (derived from C57/B6, b haplotype), but were similar for three other B lymphoma cell lines, as well as for three T lymphoma cell lines when we used monoclonal antibodies specific for L^d, D^b and K^d (data not shown). Tetramerization is therefore likely to be universal during class I assembly.

Our results indicate that MHC heavy chain/ β_2 -microglobulin complexes are post-translationally assembled into non-covalently associated tetramers which are transported to the cell surface. This finding is easy to reconcile with models of multi-valent ligand induced antigen-receptor crosslinking (as opposed to models that invoke only an allosteric change) for T-cell activation. A corollary might be that only high concentrations of MHC class I-binding peptides or peptides with a high affinity will lead to the occupancy of two or more grooves in a given tetramer. This condition is likely to be met by peptides in virally infected cells and may have important implications for models of immunological tolerance, immune surveillance and viral latency.

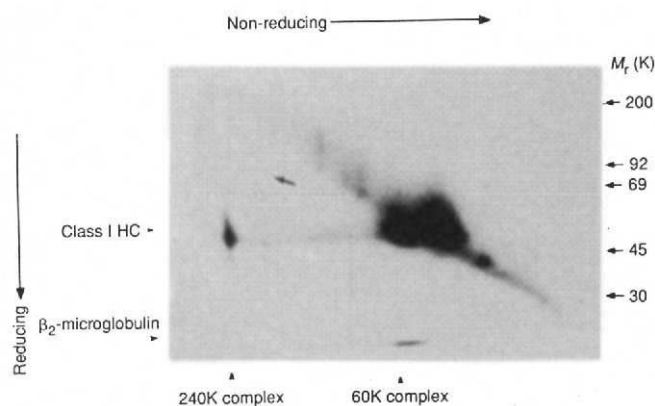


FIG. 3 Two-dimensional non-reducing/reducing analysis of metabolically labelled crosslinked anti-MHC immunoprecipitates. Cells were metabolically labelled, crosslinked with DSP, lysed and immunoprecipitated as described for Fig. 1. Analysis on a non-reducing/reducing two-dimensional polyacrylamide-SDS gel was essentially as described¹⁴; both dimensions involved 9% polyacrylamide gels, with crosslinker cleavage being generated during the mercaptoethanol reduction step. Rainbow M_r markers (Amersham) were included in the sample during the first dimension and also run in a parallel lane in the reducing dimension. Off-diagonal heavy chain and β_2 -microglobulin spots were observed in positions corresponding to the 60K and 240K complexes. Arrow indicates the position (barely visible) of the poorly labelled 88K putative chaperone in the transient 140K complex.

Intracellular class II oligomers have been described³, but the existence of such oligomers on the cell surface has not been established. Although we now have considerable knowledge about MHC class I assembly^{4,5}, the temporal order of some of the events in this process is not clear. Other issues being

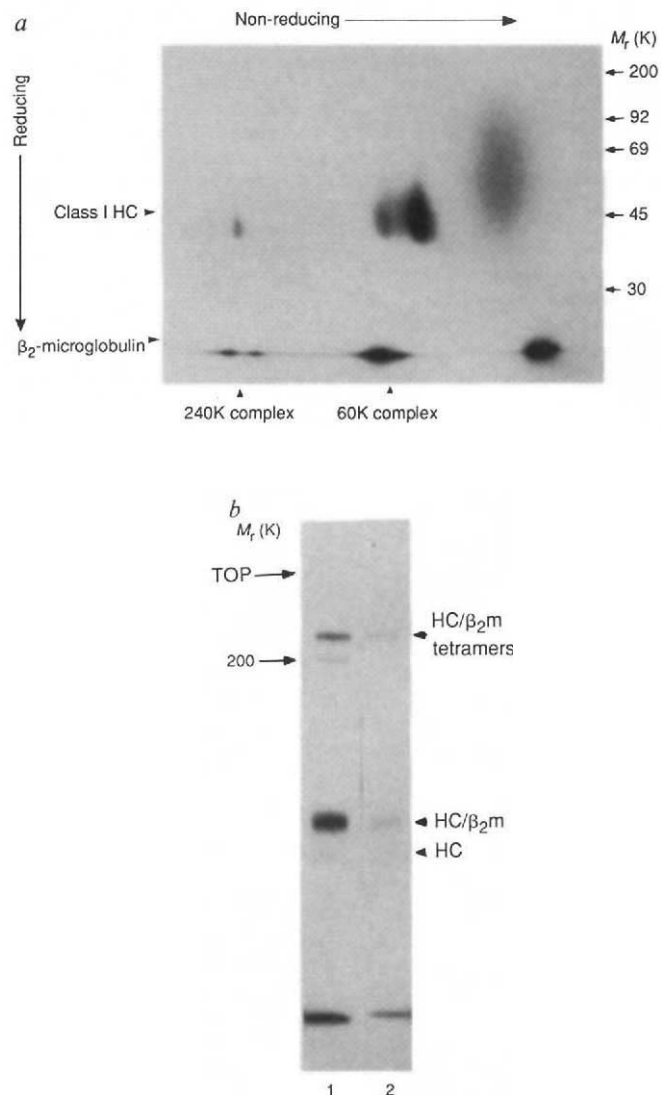


FIG. 4 Analysis of cell-surface MHC complexes following mild lysis and crosslinking. *a*, Two-dimensional analysis of crosslinked surfaces MHC complexes. 3320-11 cells, surface-iodinated as described¹⁴, were lysed and crosslinked (10^8 cells were labelled and lysed in 1.0 ml of 1.25 mM DSP, 1% digitonin; see Fig. 1 legend). The sample was analysed using the same non-reducing/reducing system as for Fig. 3. *b*, 5×10^7 3320-11 cells were iodinated and divided into two equal portions. One was immediately lysed and crosslinked ($\sim 2.5 \times 10^7$ cells in 1.0 ml 1.25 mM DSP/1% digitonin/2 mM PMSF mixture). The other portion was incubated in complete medium (RPMI with 10% FCS) for 5 h and crosslinked and lysed under identical conditions after incubation. Both samples were immunoprecipitated using the 28-14-8S antibody and analysed on a 5–15% polyacrylamide-SDS gradient gel. Positions of M_r markers are indicated. Note that in crosslinking experiments, crosslinker to cell ratios directly determine the relative proportions of 240K and 60K crosslinked species (titrations not shown). In *b* the relative crosslinker to cell ratios were 2–3-fold higher than the same ratio in *a*. Some trailing of β_2 -microglobulin is seen close to the tetrameric species in *a* and a minor faster-migrating band is seen close to the tetramer species in *b*. On two-dimensional analysis (not shown) this latter band contributes to a trailing β_2 -microglobulin species similar to that seen in *a*. This minor species could represent trimers or empty class I tetramers, but probably represents intramolecularly crosslinked β_2 -microglobulin molecules (β_2 -microglobulin is relatively lysine-rich and is prone to conformation-dependent mobility shifts on SDS-PAGE¹⁵).

addressed include the role of the 88K putative chaperone, motifs involved in tetramerization and the possible existence of tetramers containing more than one allelic species. □

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Heat shock in *Escherichia coli* alters the protein-binding properties of the chaperonin groEL by inducing its phosphorylation

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WHEN bacterial or eukaryotic cells are exposed to high temperatures or other harsh conditions, they respond by synthesis of a specific set of heat-shock proteins^{1–3}. Certain heat-shock proteins such as groEL, called 'chaperonins', can prevent misfolding and promote the refolding and proper assembly of unfolded polypeptides generated under harmful conditions^{2,4}. We report here a new aspect of the heat-shock response in *Escherichia coli*: at high temperatures a fraction of groEL becomes modified covalently, altering its interaction with unfolded proteins. The heat-modified form can be eluted with ATP from an unfolded protein more easily than normal groEL. The critical heat-induced modification seems to be phosphorylation, which is reversed on return to low temperature. Treatment of the modified groEL with phosphatases caused its apparent size, charge and binding properties to resemble those of the unmodified form. Thus during heat shock some groEL is reversibly phosphorylated, which allows its ATP-dependent release from protein substrates in the absence of its usual cofactor (groES), and probably promotes the repair of damaged polypeptides.

In *E. coli*, the groEL protein⁵ and its homologue in mitochondria, hsp60 (ref. 6), are essential for cell growth and for survival at high temperatures. The groEL protein, together with its cofactor groES, promotes the refolding of certain denatured proteins *in vitro*^{7,8}. It also binds to newly synthesized polypeptides⁹ and thus seems to be involved in the normal folding process, as well as in the refolding of damaged proteins in heat-shocked cells⁴. To study the interaction of heat-shock proteins with unfolded polypeptides, we used an affinity column containing as a ligand the fusion protein, CRAG, which contains protein A and unfolded domains^{10,11}. When expressed in *E. coli*, CRAG becomes associated with several chaperonins, including groEL and dnaK (the bacterial homologue of hsp70)¹⁰. When a radio-labelled extract of *E. coli* was loaded on a CRAG column, the

major heat-shock proteins dnaK and groEL bound selectively to different sites on CRAG (Fig. 1)¹⁰. All the bound dnaK and some groEL could be eluted by addition of ATP-Mg²⁺ (ref. 10).

When an extract from cells grown at 25 °C was loaded to this CRAG column, about 13% of the total cellular groEL bound to it, but addition of ATP released only 4–8% of this bound protein, despite repeated washing with ATP. Most of what remained on the column could be eluted with acetic acid (pH 2.5) or with ATP plus the protein cofactor, groES (M.Y.S. and A.L.G., manuscript in preparation). However, when cells were switched from 25 °C to 42 °C for 30 min, a similar amount bound to the column, but 7–10 times more groEL was eluted with ATP (Fig. 1; Table 1). The induction of heat-shock proteins at 42 °C roughly doubled the total amount of groEL in the cell (Table 1). This disproportionate increase in the amount of groEL in the ATP-eluted fraction suggests that heating the cells somehow altered some of the pre-existent chaperonin, probably by a covalent modification. Furthermore, after heat treatment, the amount of groEL not released by ATP was half that in cells

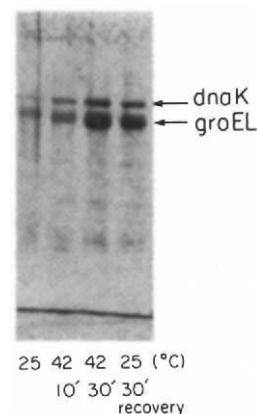


FIG. 1 Effect of growth temperature on the elution of groEL with ATP from CRAG. *E. coli* cells grown at 25 °C were shifted to 42 °C for 10 or 30 min, and then shifted back to 25 °C for 30 min. Samples were taken at the indicated time-points, extracts were passed over the CRAG column, and the bound groEL was eluted with ATP.

METHODS. To prepare the CRAG-affinity column, we expressed this recombinant protein in *E. coli* RR1Δm15. Cells were routinely grown at 30 °C in Luria broth (LB) and were then moved to 42 °C for 2 h to induce the expression of the CRAG fusion protein, because the gene is under the control of the PI promoter, which is regulated by the temperature-sensitive cl857 repressor. After collection, the cells were resuspended in 20 mM Tris, 5 mM EDTA, 1 mg ml⁻¹ lysozyme (pH 8.0), quick-frozen and thawed. The cells were then disrupted by sonication for 20 s and centrifuged at 14,000g for 10 min. Supernatant (50 ml, containing about 1 g total protein) was applied at 4 °C to a 2-ml column of horse IgG-Sepharose (Sigma) and washed with 50 ml 20 mM Tris, 150 mM NaCl, and 1 mM DTT (pH 7.8). The proteins associated with CRAG were then eluted at room temperature with 10 ml 1 mM ATP, 10 mM Mg²⁺. After elution with ATP, the IgG-Sepharose with bound CRAG (but lacking the associated heat-shock protein) was used in the binding experiments. To study the effects of heat shock on the binding of groEL to CRAG *in vitro*, *E. coli* (25 ml culture) were grown in 100 ml flasks on Minimal Salt (Davis) (Difco) with thiamine, 0.5% glucose, and all amino acids except methionine, valine and serine at 35 °C. At early log-phase, the cells were moved to 25 °C and incubated for 2 h. Growing cells (still at early log-phase) were moved to a 42 °C water bath. To shift cells back to 25 °C, the flask was transferred from 42 °C to a waterbath at 25 °C, which reduced the culture temperature to 25 °C in less than 5 min. Proteins (10 μg) from the crude cell extract were applied to a 2-ml CRAG column, washed with buffer extensively and finally eluted with 1 mM ATP. The material that remained bound to the column after elution with ATP was eluted with 100 mM acetic acid (pH 2.5). The eluted proteins were precipitated with 10% TCA, washed with acetone and separated by SDS-PAGE¹⁷. To quantitate the portion of total cellular groEL recovered after elution with ATP, we did serial dilutions of the cell extracts, and western blots with anti-groEL antibody and ¹²⁵I-labelled protein A. In parallel, we ran similar western blots of the ATP-eluted groEL, and then calculated the relative amounts of the groEL protein in these two samples.

at 25 °C. Thus, heat shock seems to promote the conversion of a portion of groEL to a form which can be eluted easily with ATP from the unfolded protein column. Under these binding conditions, CRAG must compete with endogenous proteins for binding of groEL, most of which is probably associated with nascent chains⁹ or denatured proteins¹². Therefore, this 7–10-fold change in elution properties of groEL on heat shock must enhance the amount of groEL available for interaction with damaged proteins.

This alteration did not require protein synthesis. Accordingly, when cells were shifted from 25 °C to 42 °C in the presence of chloramphenicol (100 µg ml⁻¹) to block protein synthesis, a threefold increase in the amount of ATP-eluted groEL was still seen. This change in groEL-binding properties on heat shock was rapidly reversed after reduction of the growth temperature (Fig. 1). For example, when cells growing at 25 °C were moved to 42 °C for 30 min and then returned to 25 °C for 30 min, the cellular level of groEL remained high, but 50% less groEL was eluted from the column with ATP than in extracts of cells at 42 °C.

To define how the heat-induced form of groEL eluted with ATP differs from the form eluted with acid, their mobilities were compared by SDS-polyacrylamide gel electrophoresis (PAGE). The apparent M_r of the ATP-eluted groEL was almost 60K, or 4–5K higher than that of the groEL, which was then eluted with acid (Fig. 2a, b) and which corresponded in size to the major groEL band in the cell (56K). This difference in the mobilities of fully denatured proteins indicates that the ATP-eluted form is covalently modified. Accordingly, the two bands reacted with anti-groEL antibody with similar affinities, and showed similar patterns of peptides on digestion with protease V8 (data not shown). On two-dimensional isoelectrofocusing SDS-PAGE, the ATP-eluted groEL showed a more acidic isoelectric point than the acid-eluted form (Fig. 2c). But on native gel electrophoresis, the two forms of groEL displayed similar mobilities (not shown). Thus, the multimeric state of groEL does not seem to change after heat shock.

In some experiments, ATP did not elute just the '60K' modified form of groEL. Instead, SDS-PAGE of the eluted fraction revealed either two bands of 55 and 60K, or a smear

TABLE 1 Effect of heat shock and recovery at 25 °C on the elution of groEL from CRAG by ATP

Growth conditions	Total groEL in cell (arbitrary units)	ATP-eluted groEL	
		(arbitrary units)	(% of Total)
25 °C	66	1	1.5
42 °C 10 min	138	2.5	1.8
42 °C 30 min	153	8	5.2
42 °C 30 min then 25 °C 30 min	178	5	2.8

Amounts of groEL in ATP-eluted fraction and in total cell extract were determined according to the legend to Fig. 1.

between 55 and 60K. Possibly groEL undergoes multiple modifications, and the smear corresponds to different degrees of modification, or these results may be due to rapid demodification of some of the '60K' groEL *in vitro*. We have found that ATP-eluted groEL is demodified spontaneously on storage for several hours (unpublished observations). Alternatively, modified and unmodified subunits may coexist in the groEL multimer, and the modification of only some subunits may be sufficient for its elution by ATP.

One explanation for this increase in apparent M_r and lower isoelectric point could be phosphorylation. Therefore, cells were labelled with ³²P-orthophosphate at 42 °C. Cell extract was passed over the CRAG column, and the eluted material subjected to SDS-PAGE. The ATP-eluted groEL contained some ³²P (Fig. 3a), and its specific activity was at least 10 times greater than that of the groEL that remained bound (not shown). Thus, the heat-induced form seems to be phosphorylated selectively. Purified groEL preparation exhibits groEL kinase activity (M.Y.S. and A.L.G., manuscript in preparation.)

To test if this phosphorylation is responsible for the change in groEL properties, the ATP-eluted fraction as incubated with alkaline or acid phosphatases for 15 min and then subjected to SDS-PAGE (Fig. 3b). Both enzymes caused most of the

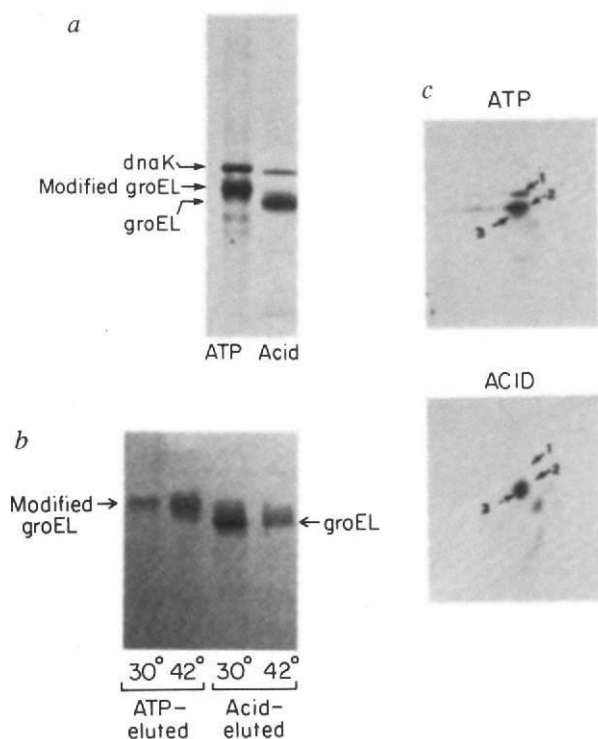


FIG. 2 a, The ATP-eluted fraction of groEL has a different apparent M_r from the acid-eluted form. The ATP-eluted and acid-eluted fractions from normal and heat-shocked cells were run on SDS-PAGE. b, Heat shock increases the amount of groEL eluted with ATP (low mobility form) and decreases the amount that remained bound to the column (high-mobility form). The ATP-eluted and acid-eluted fractions from normal and heat-shocked cells were run on SDS-PAGE followed by western blot with anti-groEL antibody. c, Two-dimensional electrophoresis of ATP- and acid-eluted fractions. 1, dnaK; 2, modified groEL; 3, unmodified groEL. Two-dimensional electrophoresis was according to Neidhardt¹⁸.

modified groEL band to shift to the position of the unmodified form. Moreover, the incubation with alkaline phosphatase altered the elution properties of the modified groEL. When the ATP-eluted fraction was reloaded immediately on a CRAG column in the presence of 5 mM EDTA, all the groEL bound and could later be eluted with ATP. Incubation of this fraction with alkaline phosphatase for 10 min before reloading did not affect binding to CRAG, but reduced the amount of the ATP-eluted groEL by 45–60%. Thus, phosphorylation seems responsible for the heat-induced elution properties. (The incomplete reversal of this behaviour by phosphatase was probably due to the incomplete dephosphorylation of the groEL multimer.)

The heat-shock response has been viewed as a marked alteration in gene transcription and translation that improves the organism's ability to withstand harsh conditions. This study demonstrates a new aspect of this phenomenon: that heat shock also alters the ability of groEL to interact with and dissociate from unfolded proteins. On moving cells to 42 °C, the other major chaperonin in *E. coli*, dnaK (the hsp70 homologue), is also phosphorylated, which enhances its binding to unfolded proteins (M.Y.S. and A.L.G., manuscript in preparation). Thus, heat shock causes alterations in the functional properties of multiple chaperonins. It remains to be established whether the homologous heat-shock proteins in eukaryotic cells or other heat-shock proteins in *E. coli* also undergo temperature-induced phosphorylation.

This phosphorylation of groEL at high temperature and its reversal at low temperature must result in some physiological advantage to the organism. Most probably, the heat-modified form is more active or more efficient in promoting the refolding of damaged proteins than the major groEL species. Accordingly, the phosphorylated form, unlike the major form of groEL, does not require groES to dissociate from our model unfolded protein.

It thus may have an altered ATPase activity or catalyse novel chaperonin functions¹².

It is possible that these heat-induced alterations in groEL and dnaK contribute to the phenomenon of 'acquired thermotolerance', in which cells become resistant to normally lethal temperatures on mild heat shock. Although heat-shock proteins are essential for acquired thermotolerance, this state can be reached without new protein synthesis^{13–16}. Therefore, the heat-shock-dependent modifications of groEL and dnaK could be the critical adaptations conferring this acquired thermotolerance. □

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Protein–DNA interactions at a yeast replication origin

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AN understanding of the protein–DNA interactions *in vivo* at origins of DNA replication in eukaryotes is essential to delineate the mechanism of initiation of DNA synthesis and its control in the cell cycle^{1,2}. In the yeast *Saccharomyces cerevisiae*, a family of sequences known as autonomously replicating sequences (ARs) function as origins of bidirectional DNA replication on plasmids and, in several instances, also in their normal chromosomal location³. Here we use nucleotide resolution genomic footprinting to investigate the association of proteins with ARS1. Nuclease protection patterns indicate that at least two different cellular factors interact with functional elements in ARS1. The first seems to be ARS-binding factor 1. The second seems to be a novel protein that generates extensive protection over the essential ARS consensus sequence and phased DNaseI-sensitive sites across a functionally important flanking sequence. Hypersensitivity of this region to cleavage by copper phenanthroline indicates that it is under torsional strain, analogous to that produced at transcriptional start sites by assembly of an initiation complex. The protection *in situ* is similar to that generated by the origin recognition complex (ORC) protein.

All yeast ARs contain an exact or close match to the core consensus sequence, 5'-A_TTTTAT_GTTT_T-3' and this sequence seems essential for origin function. In addition to this element, flanking sequences are important for efficient ARS activity^{1,2}. At ARS1, the core consensus sequence lies in a region called domain A and the flanking sequence is known as domain B (ref. 4). Domain B has recently been divided into three

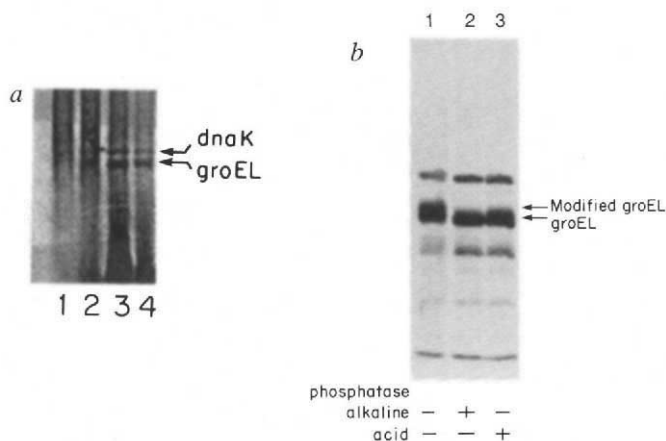


FIG. 3 *a*, The ATP-eluted form of groEL from ³²P-labelled cells is phosphorylated. *b*, Effect of phosphatase treatment on the mobility of modified groEL. Before ATP addition (lane 1); sequential fractions (1 ml) eluted with ATP (lanes 2, 3, 4). Lanes 1, No phosphatase treatment; 2, alkaline phosphatase; 3, acid phosphatase.

METHODS. *a*, For labelling with ³²P-orthophosphate, MPH86 cells were grown overnight on LB at 30 °C, then washed twice with the following medium: 12 g Tris, 2 g NH₄Cl, 2 g KCl, 0.5 g MgCl₂, 200 mg Na₂SO₄, 5 mg thiamine, 3 g glycerol, 1.5 g casamino acids per litre. Cells were resuspended in the same medium supplemented with 10⁻⁶ M K₂HPO₄ and 1 mCi ³²P-orthophosphate (Amersham). Cells were incubated at 35 °C for 1 h, then shifted to 25 °C for 1 h, and finally incubated at 42 °C for 40 min. Extracts from labelled cells were prepared, passed through the CRAG column, and groEL and dnaK were eluted with ATP. *b*, Cells were labelled with ³⁵S as for Fig. 2*a*. ATP-eluted fraction (1 ml) was incubated with 10 units alkaline phosphatase from calf intestine (Boehringer) at 37 °C for 10 min. For treatment with acid phosphatase, pH of 1 ml ATP-eluted fraction was adjusted to 6.2 with 1 M MES buffer and incubated at 37 °C for 10 min with 10 units acid phosphatase from potato (Sigma). Then protein was precipitated with trichloroacetic acid and resolved on SDS-PAGE.

functional subdomains designated B1, B2 and B3 (ref. 5). Several proteins have been identified with specificity in their binding to ARS DNA. The best characterized of these is the multifunctional ARS-binding factor 1 (ABF1)⁶⁻⁹. A binding site for ABF1 lies in B3 (ref. 7) and results from detailed mutagenesis of this site are consistent with ABF1 having a role in ARS function⁵. The possible role of other DNA-binding proteins¹⁰⁻¹⁴ in ARS function have been less well characterized and the ultimate function of domains A, B1 and B2 remains unclear.

To clarify the situation and identify new proteins, we used the technique of nucleotide resolution genomic footprinting¹⁵ to examine protein-DNA interactions at ARS1. Briefly, cells are lysed and immediately treated with nuclease. The position of cleavage sites is then determined by several rounds of primer extension with *Taq* DNA polymerase using a 5' end-labelled oligonucleotide complementary to a sequence outside ARS1. Cleavage sites in naked genomic DNA digested to a similar extent are visualized in the same manner and serve as a control.

We have focused our initial efforts on examining ARS1 in the 1.4-kilobase (kb) TRP1-ARS1 circle. In this plasmid, ARS1 is free of nucleosomes¹⁶⁻²⁰ and next to the ARS consensus sequence is a tightly positioned nucleosome^{16,18,20}. Several of these studies have suggested the binding of non-nucleosomal proteins in ARS1 (refs 16-19), although the resolution of the techniques used was not sufficient to describe the interactions in detail. ARS1 chromatin was probed by digestion with DNaseI (Fig. 1). The B3 element on both strands (Fig. 1a, b) was protected from DNaseI digestion. Because the extent of this protection is very similar to that seen with purified ABF1 (ref. 7), we conclude that ABF1 or an ABF1-like protein is bound *in vivo* in the B3 element of ARS1.

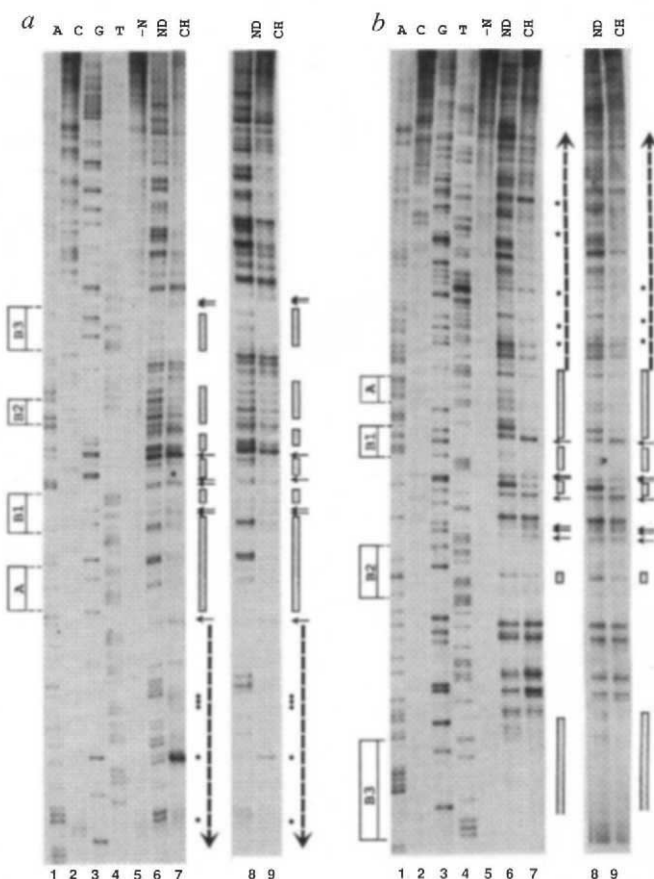
Sequences in the positioned nucleosome outside domain A (refs 16, 18, 19, 20) exhibited a 10-base-pair (bp) periodicity of DNaseI cleavage, best seen at the bottom of Fig. 1a, lanes 7 and 9, indicated by filled circles. Because the DNA in nucle-

somes is only accessible to DNaseI digestion at 10-bp intervals²¹, these results strongly indicate that this nucleosome is precisely positioned in a large proportion of molecules. Protection distal to B3 (top of Fig. 1a) is presumably due to the nucleosome on the TRP1 side of ARS1 (refs 16-19). Domain A, containing the 11-bp ARS consensus sequence, lies in a region protected from DNaseI digestion on both strands. A series of short protected regions separated by sites of equal or enhanced cleavage at about 10-bp intervals extend from domain A to B2 and a slightly larger region around B2 was also protected preferentially on one strand (Fig. 1a). Band intensities were quantified by densitometric scanning of autoradiograms and results of this pattern across A and B1 are shown in Fig. 2. Protection of domain A is usually 70-80%.

Copper phenanthroline is a chemical cleavage reagent that is an informative probe to compare with DNaseI (ref. 22). Sequences both in domain A and in B3 were protected from cleavage by copper phenanthroline (Fig. 3a, b), although domain A showed preferential protection on the T-rich strand. Additionally, sequences across B1 showed a heightened reactivity to copper phenanthroline. The periodic nature of the DNaseI cleavage together with the complete accessibility to copper phenanthroline indicate that the DNA across B1 is wrapped around the outside of a protein core with the minor groove exposed to the solvent²². Figure 3c demonstrates that this pattern of copper phenanthroline protection and hypersensitivity can be seen across a wide range of digestion.

A summary of the DNaseI and copper phenanthroline cleavage sites on both strands is shown in Fig. 4. A number of different proteins bind at or around domain A *in vitro*¹⁰⁻¹⁴. The pattern of protections and hypersensitive sites across domains A and B shown here is remarkably similar to that seen *in vitro* by Bell and Stillman¹⁴ for the protein designated ORC (origin recognition complex; Fig. 4a) and strongly suggests that this factor is bound at ARS1 *in vivo*. Although the two patterns are

FIG. 1 Genomic footprinting of both strands (a, b) of ARS1 with DNaseI. Lanes A, C, G and T refer to dideoxynucleotide sequencing reactions done directly on genomic DNA; -N, undigested DNA; ND, naked DNA treated with DNaseI; CH, chromatin treated with DNaseI. Lanes 1-7 are footprints of the TRP1-ARS1 circle and lanes 8-9 are footprints on the related plasmid YRp7. METHODS. Mid-log phase SP1 (*MATa ura3-52 his3 trp1-289 leu2-2.113 ade8 can1*)²⁹ carrying the 1.4-kb TRP1-ARS1 circle or CG378 (*MATa ura3-52 trp1-289 leu2-2.113 ade5 can1*)³⁰ carrying the 5.8-kb YRp7 plasmid³¹ grown under selection were processed for genomic footprinting as described¹⁵, with the following exceptions. All buffers used during spheroplast formation contained synthetic complete medium (SCM)-tryptophan³² and cells were lysed in buffer containing 10 mM Tris HCl, pH 7.5, 10 mM MgCl₂, 5 mM 2-mercaptoethanol, 1 mM phenylmethylsulphonyl fluoride, 2 µg ml⁻¹ leupeptin and 1 µg ml⁻¹ pepstatin. The oligonucleotides used were, in a, 5'-GCGGTGAAATGGTAAAGTCAACCCCTGCG-3', and in b, 5'-GGTGTGATGTAAGCGGAGGTGTGGAGAC-3', which are complementary to ARS1 sequences on either side. Oligonucleotides were 5'-end labelled³³, extended with *Taq* polymerase in five cycles of 1 min at 94 °C and 5 min at 72 °C in reactions that contained 30 µg DNA and processed as described¹⁵. Sequencing reactions using *Taq* polymerase and end-labelled oligonucleotides were done as described¹⁵. Samples were subjected to electrophoresis in 5% sequencing gels, dried and autoradiographed. The positions of domain A and the three subdomains of domain B (B1, B2, B3)⁵ are shown to the left of each panel. To the right of each panel the regions of protection are shown as grey boxes and nucleotides of equal or enhanced cleavage are indicated as arrows. Filled circles to the right of each panel indicate sites of equal or enhanced DNaseI cleavage lying in the nucleosome adjacent to domain A (refs 16, 18, 19). The position of this nucleosome^{16,18} is shown as a thick broken arrow.



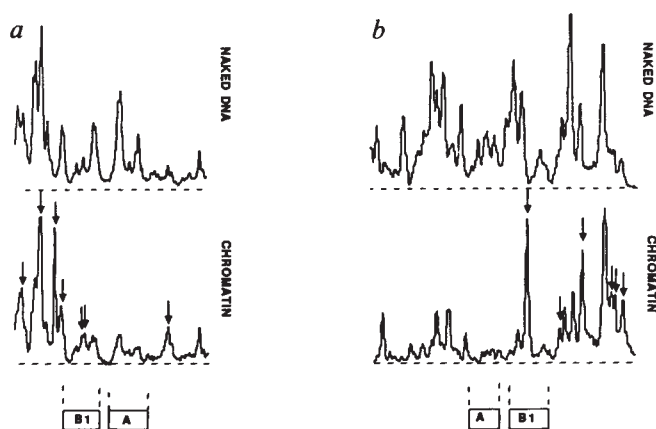


FIG. 2 Quantification of protections across domains A and B1 for each strand (a, b). Band intensities in lanes 5, 6 and 7 from Fig. 1a and b were determined by scanning the autoradiograms with a Molecular Dynamics computing densitometer model 300B using ImageQuant software. Shown plotted are the results of these scans for naked DNA and chromatin after subtracting the background from the equivalent position in the -N lane. The positions of equal or enhanced cleavage in chromatin are indicated with arrows. The baseline is indicated as a horizontal dashed line.

nearly identical across domains A and B1, they diverge around B2. ORC can bind to B2, a 9/11 match to the ARS consensus sequence, *in vitro* only in the absence of a functional domain A¹⁴. Therefore, the protection seen here over domain B2 is either a separate, unidentified factor or, in the context of chromatin, and perhaps in cooperation with ABF1, ORC can bind to both domain A and B2 simultaneously.

These patterns are very similar to those generated by transcription complexes assembled at the adenovirus major late promoter *in vitro*. Binding of a partially purified preparation of the transcription factors TFIID and USF to this promoter generates a large region of DNaseI protection over the TATA box and a region of 10-bp periodic protections and enhancements that spans the start site of transcription²³. Moreover, on assembly of the complete initiation complex, the transcription start site

undergoes a conformational change and becomes hypersensitive to copper phenanthroline²⁴, consistent with the introduction of torsional strain. This step does not require ATP hydrolysis²⁴ and may precede the formation of an open complex²⁵. By analogy, ORC may function by specifically recognizing the ARS consensus sequence and generating torsional strain across domain B. Such torsional strain may aid in the formation of an open complex in ARS1 containing unwound DNA similar to those produced by other DNA replication initiator proteins²⁶⁻²⁸. We have so far been unable to detect single-stranded regions in ARS1 using chemical and enzymatic probes (data not shown), indicating that although this region may be under torsional strain, it is still largely double-stranded.

The DNaseI protections seen at domains A and B3 in this report are nearly 80%, suggesting that both ORC and ABF1

FIG. 3 Genomic footprinting of ARS1 with copper phenanthroline (OP-Cu). a, b, OP-Cu footprints at optimal levels of digestion for each strand of ARS1 as in Fig. 1. c, Position of OP-Cu cleavage sites across a range of digestion. Cells were treated and extracts prepared as described in Fig. 1. Copper phenanthroline digestions were done by a modification of published methods (ref. 22). Briefly, after cell lysis, 30 μ l copper phenanthroline (0.45 mM CuSO₄, 2 mM 1,10-phenanthroline) and various amounts of hydrogen peroxide from 0.2 to 10 mM were added to 0.3 ml cell extract prepared as described above. After 30 s, reactions were stopped by the addition of 15 μ l each of 2 M thiourea and 0.12 M 2,9-dimethylphenanthroline and processed as described¹⁵. Conditions for *Taq* polymerase extension, processing and symbols were as for Fig. 1, except that the region of general enhanced sensitivity to OP-Cu across B1 is indicated as a thick black bar bounded by arrows. The average length of genomic DNA in c as assessed by agarose gel electrophoresis ranged from >8 kb in lane 2 to <0.7 kb in lane 5. Lanes 3 and 4 of b show a repeat of the experiment in lanes 1 and 2.

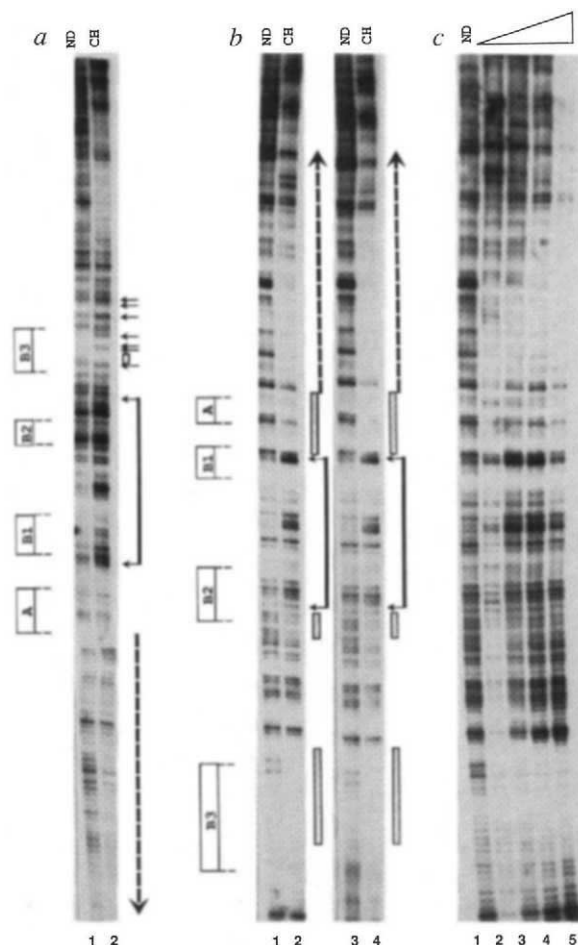
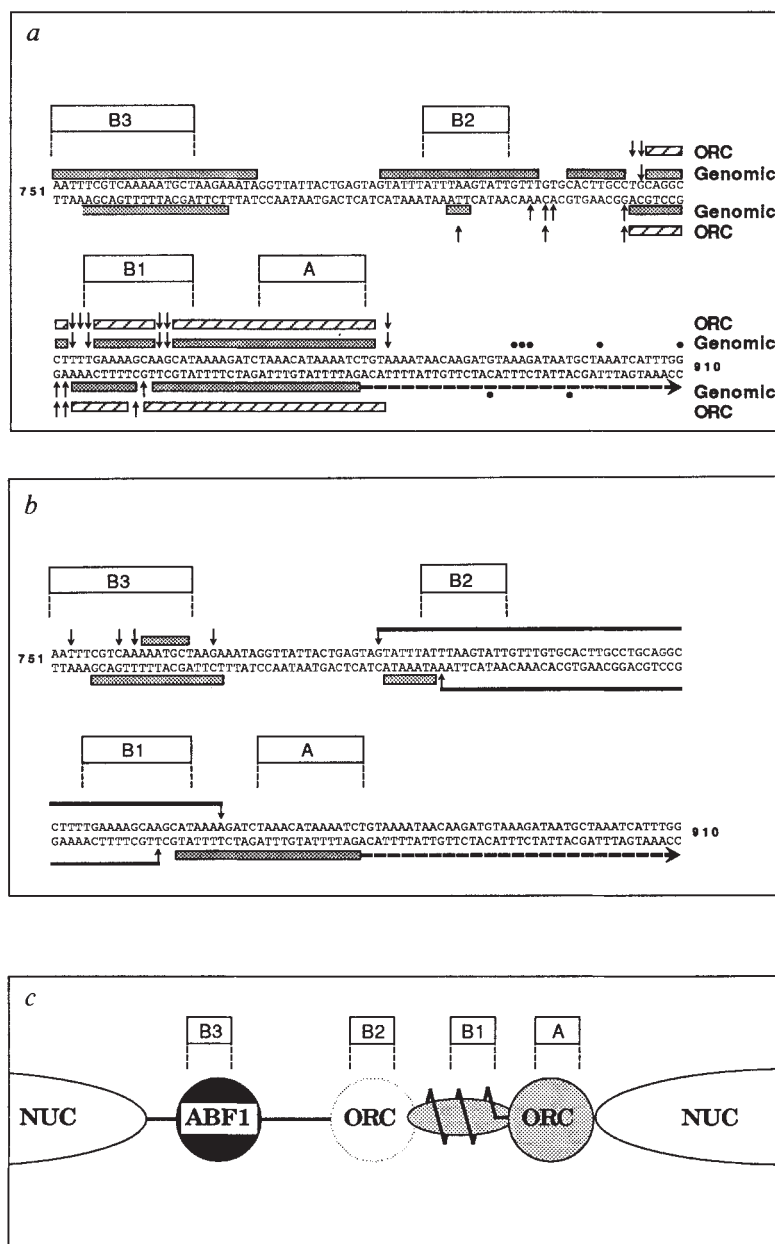


FIG. 4 Summary of genomic footprinting. The positions of protections and enhanced cleavage sites as shown in Figs 1 and 2 are summarized here for DNaseI (a) and OP-Cu (b). Symbols are as in Figs 1 and 3. The positions of the protections and hypersensitivities generated by purified ORC at ARS1 as determined by Bell and Stillman¹⁴ are shown above and below the positions of the genomic footprints in striped boxes. Note that when converting the data in Figs 1, 2 and 3 into Fig. 4, two considerations were made. First, because the sequencing reaction products terminate in a dideoxynucleotide, they migrate about 1/2 nucleotide faster than the corresponding deoxynucleotide terminated product. Second, cleavage sites lie on the complement of the sequenced strand. In c, the most likely explanation for the protection patterns is shown. In this model, domain A is bordered by a tightly positioned nucleosome, B3 is bound by ABF1 and A and B2 are bound by ORC, although the binding to B2 seems much weaker. ORC binding extends over B1 with the DNA in this region wound around the outside of a protein core and under torsional stress.



may be bound at ARS1 during most or all of the cell cycle. If this is true, then the control of the initiation of DNA replication may occur at levels other than simply regulating the availability of these origin-binding factors. To our knowledge, this study is

the first use of high-resolution genomic footprinting to present a detailed analysis of protein-DNA interactions at an origin of replication and the technique should be useful in the analysis of replication origins in other systems. □

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Self-splicing introns in tRNA genes of widely divergent bacteria

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THE organization of eukaryotic genes into exons separated by introns has been considered as a primordial arrangement^{1,2} but because it does not exist in eubacterial genomes it may be that introns are relatively recent acquisitions³. A self-splicing group I intron has been found in cyanobacteria at the same position of the same gene (that encoding leucyl transfer RNA, UAA anticodon) as a similar group I intron of chloroplasts^{4,5}, which indicates that this intron predates the invasion of eukaryotic cells by cyanobacterial endosymbionts. But it is not clear from this isolated example whether introns are more generally present in different genes or in more diverse branches of the eubacteria. Many mitochondria have intron-rich genomes and were probably derived from the alpha subgroup of the purple bacteria⁶ (or *Proteobacteria*⁷), so ancient introns might also have been retained in these bacteria. We describe here the discovery of two small (237 and 205 nucleotides) self-

splicing group I introns in members of two proteobacterial subgroups, *Agrobacterium tumefaciens* (α) and *Azoarcus* sp. (β). The introns are inserted in genes for tRNA^{Arg} and tRNA^{Leu}, respectively, after the third anticodon nucleotide. Their occurrence in different genes of phylogenetically diverse bacteria indicates that group I introns have a widespread distribution among eubacteria.

Group I introns capable of self-splicing *in vitro* can be detected in RNA extracts by end-labelling with [³²P]GTP, a cofactor that becomes covalently bound to the 5' end of the intron during its excision⁸. We screened deproteinized extracts from 14 genera belonging to *Proteobacteria*, two of which provided evidence for group I introns. Single labelled RNA species of ~200 and 230 nucleotides were obtained from *Azoarcus* BH72 and *A. tumefaciens* A136, respectively (Fig. 1a and b, lanes 1). The latter strain is cured of the Ti plasmid⁹, which is involved in crown-gall formation in dikotyledonous plants, and belongs to the α -subgroup⁷. *Azoarcus* sp. is a nitrogen-fixing, grass-root-associated heterotroph which belongs to a new genus in the β -subgroup (B.R.-H. *et al.*, manuscript in preparation). The phylogenetic relationship of major eubacterial groups is shown in Fig. 1c.

We isolated genomic clones of both *Azoarcus* BH72 (Fig. 1a) and *A. tumefaciens* (Fig. 1b), whose *in vitro* transcripts are self-splicing. When plasmid inserts were transcribed with T3 RNA polymerase, products appeared in addition to the expected run-off transcript (lanes 2). Their sizes were consistent with the intron signals seen in GTP-labelling assays (LI, lanes 1), and the run-off transcripts shortened by excision of the intron (LE). After further incubation at temperatures favourable for splicing, run-off transcripts diminished and splice products accumulated

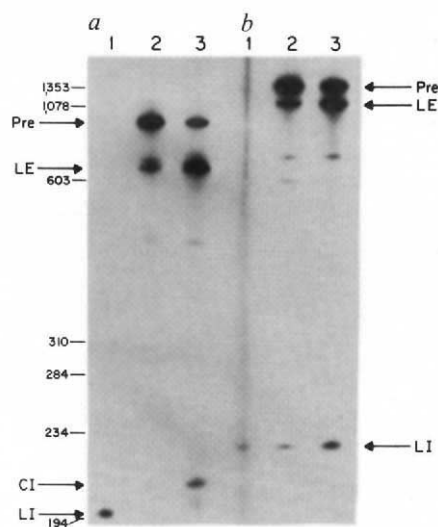
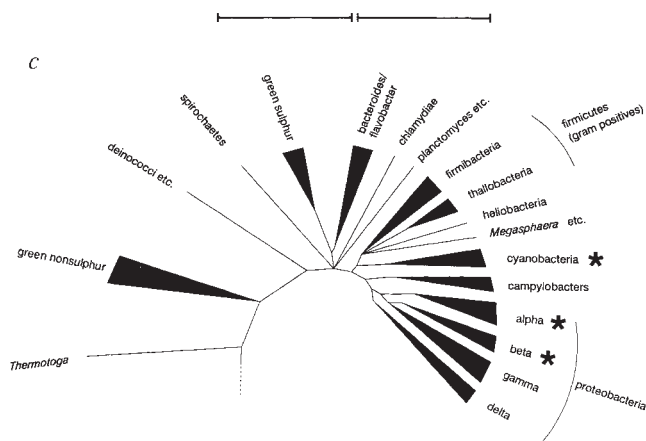


FIG. 1 Self-splicing of introns from a, *Azoarcus* BH72 and b, *A. tumefaciens* A136. Lane 1, end-labelling of *in vivo* RNA with [α -³²P]GTP; lane 2, *in vitro* transcription of genomic clones for 20 min at 37 °C; lane 3, samples from lane 2 were further incubated for 1 h at 50 °C (a) or 43 °C (b), temperatures determined to be optimal for splicing in the GTP-labelling assay. Labels indicate positions expected for the run-off transcript (Pre), ligated exons (LE), linear intron (LI), and an alternative (presumably circular) form of the intron (CI). Reaction products were separated on a 5% polyacrylamide-8M urea gel. The autoradiogram is shown. Sizes of DNA markers are in nucleotides (left). c, Principal phylogenetic groups of the eubacteria, based on 16S ribosomal RNA sequences²³ (not strictly to scale; adapted from ref. 24). Groups that have been shown to harbour group I introns in tRNA genes are indicated by an asterisk.

METHODS. Group I intron end-labelling: 5.3 μ g deproteinized RNA was incubated with [α -³²P]GTP²⁵ for 1 h at 50 °C (a) or 43 °C (b). *In vitro* transcription: plasmids pBGB1.1 (*Sph*I insert of 700 base pairs) and pBGA12.17 (*Pst*I insert of 1.7 kilobases) were linearized with *Sst*I in the multiple cloning site or with *Xho*I 1.3 kb downstream of the T3 promoter, respectively. Template (0.1 μ g) was transcribed with T3 RNA polymerase in the presence of [α -³²P]UTP (ref. 25). GTP in the transcription mixture allows group I intron self-splicing. Cloning of intron-containing genes: assuming that an intron might be inserted into tRNA^{Leu}(UAA) genes as in cyanobacteria, we attempted to amplify these genes with degenerate primers (see Fig. 3) by the polymerase chain reaction (PCR). The only amplification product obtained from genomic DNA of *E. coli* JM109 was the size expected for tRNA (<100 bp). For *Azoarcus* BH72, however, there were two main species (about 900 bp and <100 bp) as well as a number of minor fragments (to be published elsewhere). A minor amplification product of the size expected for a 200-bp intron inserted into tRNA (~260 bp) was agarose-gel purified, reamplified and cloned (pBPB3.3). We used pBPB3.3 to prepare a ³²P-labelled probe for colony and Southern hybridizations, to detect and subclone the genomic sequence of *Azoarcus* BH72 from a pUC19 library of partially *Sau*3A-digested DNA (B.R.-H. *et al.*, manuscript in preparation). A plasmid containing a 15-kb insert hybridized to the probe, and an *Sph*I fragment of 700 bp, subsequently found to span the entire exon and intron sequences, was subcloned (pBGB1.1). As no fragment of appropriate size PCR-amplified from *A. tumefaciens* DNA with tRNA^{Leu} primers, we used the same *Azoarcus* intron probe (pBPB3.3) for heterologous hybridization. Low-stringency Southern hybridization (at 55 °C without formamide) of a genomic *Pst*I digest detected a single hybridizing fragment of 1.7 kb, which was cloned as pBGA12.17. pBSM13⁺ (Stratagene) was used for all cloning.



(lanes 3). The intron species that accumulates in *Azoarcus* had a lower electrophoretic mobility than the GTP-labelled species. Its mobility relative to other RNAs was further decreased when samples were gel-fractionated at double ionic strength (data not shown), indicating that it may represent a circular form of the intron¹⁰.

Nucleotide sequences of the *Azoarcus* and *Agrobacterium* introns are given in Fig. 2a. Both can be folded into a secondary structure (shown for *Azoarcus* in Fig. 2b) typical of group I introns. All elements common to group I introns are present^{11,12}: base-pairing regions (P1–P10), conserved sequence elements in the catalytic core (P, Q, R, S), and G and U residues preceding the 3' and 5' splice site, respectively. The latter forms a U-G base pair with the internal guide sequence in P1. Splice junctions were assigned from these features and from folding of flanking exon sequences (Fig. 3) and were confirmed by sequencing of the ligated exon species obtained after splicing *in vitro*. Both introns can be assigned to subgroup IC3 (ref. 12). They are closely related to each other and to the cyanobacterial tRNA introns (fig. 2a), the *Azoarcus* intron showing 65% sequence identity with the *Agrobacterium* intron and 53–56% with cyanobacterial introns.

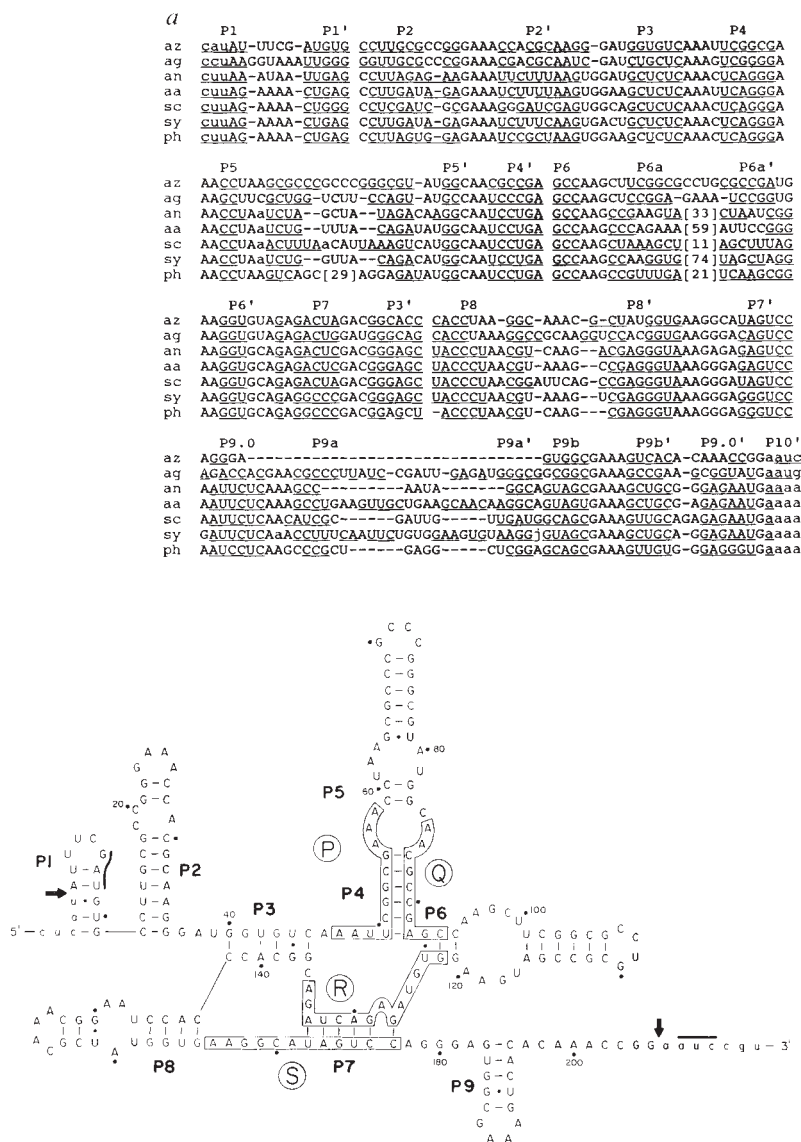
The *Azoarcus* intron is remarkably short. At 205 nucleotides, it is the smallest naturally occurring group I intron so far described as self-splicing *in vitro*. Optional stem-loop structures

like P2.1, P9.1 and P9.2 (ref. 12) are not present; all terminal loops are tetraloops, three belonging to the GNRA or UNCG families which confer unusual stability¹³. This compactness might be useful for structural studies on self-splicing introns.

Both introns are inserted into genes for tRNAs (Fig. 3). In contrast to cyanobacteria, tRNA^{Leu} (UAA) genes of *Azoarcus* BH72 (Fig. 3a) were not interrupted by intervening sequences. The *Agrobacterium* exons clearly code for tRNA^{Arg} (CCU) (Fig. 3c), but identification of the *Azoarcus* gene is not as straightforward. The *Azoarcus* exons can be folded into a tRNA with CAU anticodon (Fig. 3b), the U in the anticodon preceding the intron as in *Agrobacterium*. CAU anticodons appear in three tRNAs, for methionine initiator (i) and elongator (m) as well as isoleucine. The C at the wobble position of the isoleucine anticodon is modified to assure pairing only with the AUA codon¹⁴. Comparison of our sequence with tRNAs from *Escherichia coli* (the closest relative with data available) revealed the least mismatch with tRNA^{Ile} (twenty), followed by tRNA^{Met} (twenty-seven). Similar conclusions were drawn from examination of consensus sequences from eubacterial tRNAs (ref. 15). Although the major identity element of tRNA^{Met} is the anticodon¹⁶, all eubacterial tRNAs^{Met} share a common feature: the third base pair of the acceptor stem is pyrimidine 3:G70 (ref. 10). Changing C3:G70 of *E. coli* tRNA^{Met} to G3:C70 reduced the activity of methionyl tRNA-synthase by 6–7 fold¹⁷. Therefore, as our

FIG. 2 Intron sequence and secondary structure. a, Sequence alignment of group I introns in eubacterial tRNA genes. Introns in tRNA^{Ile} of *Azoarcus* BH72 (az) and tRNA^{Arg} of *A. tumefaciens* A136 (ag) are aligned¹² with tRNA^{Leu} introns from cyanobacteria: an, *Anabaena* PCC7120 (the genomic sequence is presented (M.-Q. Xu and D.A.S., manuscript in preparation); the published sequence⁵ from PCR clones had two uncertain assignments); aa, *Anabaena azollae*⁵; sc, *Synechococcus* (*Anacystis*) R2 (ref. 4); sy, *Scytonema* PCC7110 (ref. 4); ph, *Phormidium* N182 (ref. 4). Phylogenetically conserved base-paired regions (P1–P10) (refs 11, 12) are indicated, with Watson-Crick base pairs underlined. Numbers in brackets indicate sequence omitted. Intron sequence is in upper case, with exceptions indicating consecutive nucleotides: a, AA; j, CA. b, Secondary structure of the *Azoarcus* intron. Exon sequences are in lower case, intron in upper case letters, with arrows indicating the splice boundaries. Numbering starts with the 5' end of the intron. Conserved sequence elements of group I introns (P, Q, R, S)¹¹ are boxed. P1–P10 refer to phylogenetically conserved base-pairing regions, with the nucleotides potentially involved in P10 overlined^{11,12}.

METHODS. Complete sequences were determined from both strands of genomic clones by the dideoxy chain termination method with T7 DNA polymerase (Sequenase, US Biochem.). The *Azoarcus* sequence was determined from an intron-internal *Sma*I site, using subclones pBGB1.7 (0.65 kb *Sma*I/*Pst*I fragment) and pBGB1.10 (1.05 kb *Sma*I/*Pst*I fragment) in pBSM13⁺ (Stratagene) with primers complementary to vector sequences. The *Agrobacterium* sequence was determined from an intron-internal *Hind*III-site using subclones pBGA12.2 (1.0 kb *Hind*III/*Pst*I fragment) and pBGA12.7 (0.6 kb *Hind*III/*Pst*I fragment) in pBSM13⁺ with the same primers. The sequences of the second strands were obtained from plasmids pBGB1.1 and pBGA12.17 (containing the complete intron and exon sequences) with synthetic primers annealing to cloned DNA beyond the tRNA exons.



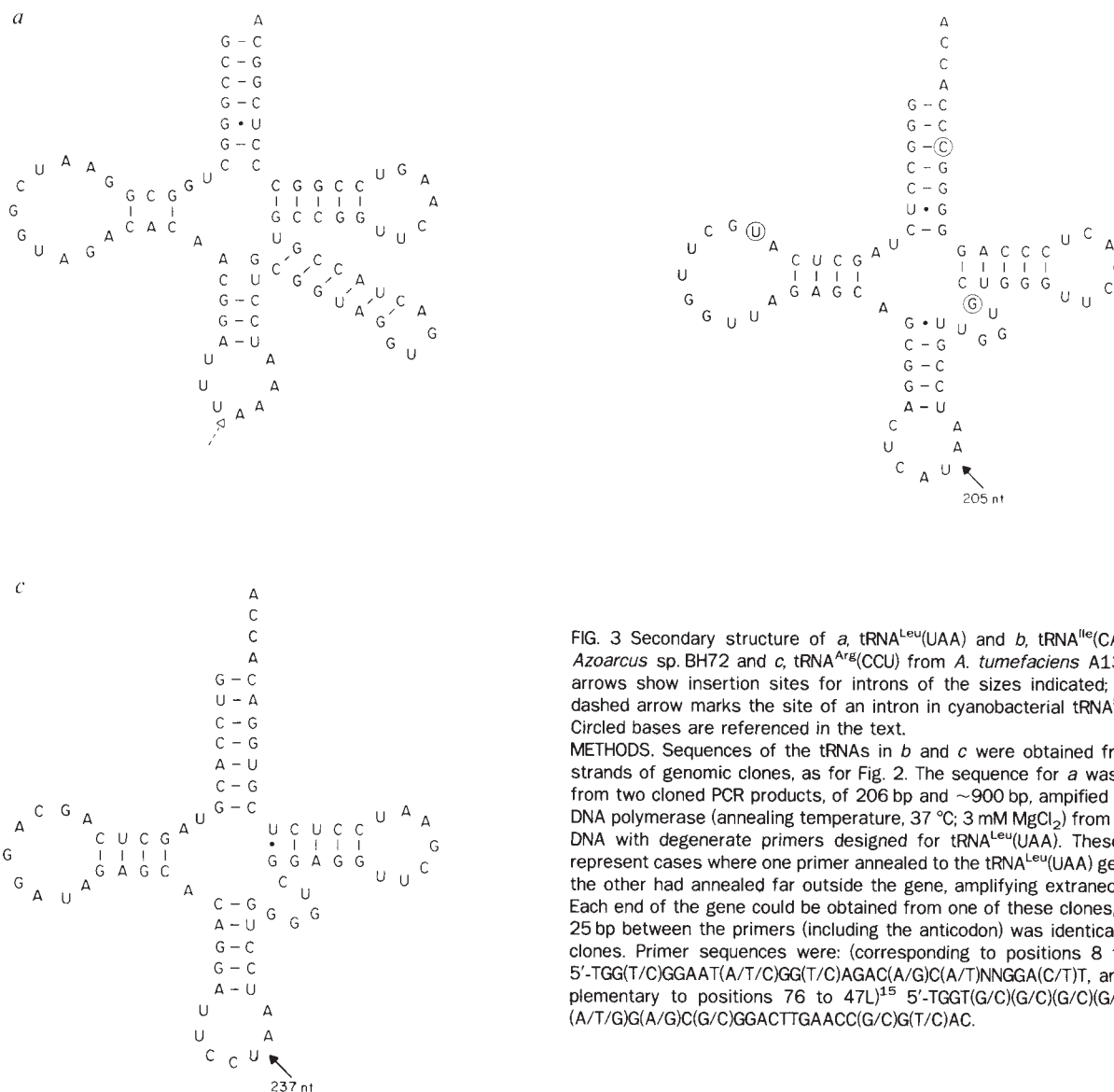
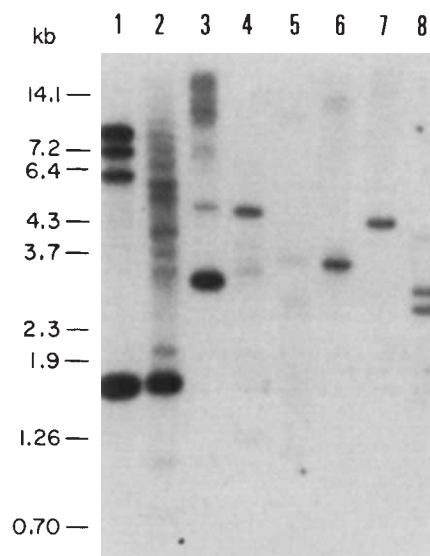


FIG. 3 Secondary structure of *a*, tRNA^{Leu}(UAA) and *b*, tRNA^{Ile}(CAU) from *Azoarcus* sp. BH72 and *c*, tRNA^{Arg}(CCU) from *A. tumefaciens* A136. Solid arrows show insertion sites for introns of the sizes indicated; an open dashed arrow marks the site of an intron in cyanobacterial tRNA^{Leu}(UAA). Circled bases are referenced in the text.

METHODS. Sequences of the tRNAs in *b* and *c* were obtained from both strands of genomic clones, as for Fig. 2. The sequence for *a* was derived from two cloned PCR products, of 206 bp and ~900 bp, amplified with *Taq* DNA polymerase (annealing temperature, 37 °C; 3 mM MgCl₂) from genomic DNA with degenerate primers designed for tRNA^{Leu}(UAA). These clones represent cases where one primer annealed to the tRNA^{Leu}(UAA) gene while the other had annealed far outside the gene, amplifying extraneous DNA. Each end of the gene could be obtained from one of these clones, and the 25 bp between the primers (including the anticodon) was identical in both clones. Primer sequences were: (corresponding to positions 8 to 33)¹⁵ 5'-TGG(T/C)GGAAT(A/T/C)GG(T/C)AGAC(A/G/C)(A/T)NNGGA(C/T)T, and (complementary to positions 76 to 47L)¹⁵ 5'-TGGT(G/C)(G/C)(G/C)(A/G)-(A/T/G)(G/A/G)(G/C)GGACTTGAACC(G/C)G(T/C)AC.

FIG. 4 Southern hybridization of the *Azoarcus* tRNA^{Ile} intron to genomic DNA of selected *Proteobacteria*. After digestion with *Pst*I, 3 µg DNA was applied per lane, except in lanes 3 (*Nsi*I + *Eco*RI) and 6 (*Eco*RI). Lane 1, *Azoarcus* sp. BH72; 2, *A. tumefaciens* A136; 3, *Neisseria gonorrhoeae* 707828; 4, *Nitrosomonas europaea*; 5, *Hydrogenophaga palleroni* RH2; 6, *Rhodospirillum rubrum* S2; 7, *Azospirillum halopraeferens* Au5; 8, *Xanthomonas campestris* pv. *Barbarae* LMG547. Sizes are given in kb. Strongest hybridization was obtained with the 1.7-kb *Pst*I fragment carrying the entire homologous group I intron (lane 1). For *Agrobacterium* (lane 2), the chief hybridization signal was due to an intron of 65% sequence identity. A signal of equal strength was obtained for *Neisseria* (lane 3); other specific signals were weaker, indicating less sequence homology. In *Azoarcus* (lane 1), three additional fragments hybridized about as strongly as the main band in lane 2. As these signals were not retained after high-stringency washes (65 °C, 0.1 × SSC), they did not result from incomplete digestion of genomic DNA, but might represent additional introns in *Azoarcus*.

METHODS. An exon-free probe was obtained by first subcloning the PCR product (in pBPB3.3) containing the *Azoarcus* intron into the *Eco*RV site of an unrelated vector, pACYC184 (pBPBAC3.3), and amplifying the complete intron by PCR. The amplified fragment was purified from an agarose gel and used as a template for radiolabelling with [α -³²P]CTP by random priming. Hybridizations were carried out at low stringency at 50 °C in 6 × SSC without formamide overnight, and washed at 50 °C in 6 × SSC.



sequence has a G3:C70 pair (Fig. 3b), it is probably a tRNA^{Ile}.

Although it follows most of the rules for tRNA structure, this structure has one unusual feature (Fig. 3b). A very highly conserved purine-pyrimidine tertiary base pair, between position 15 and the last base of the extra arm, is a pyrimidine-purine pair in this sequence, a feature shared by only a few other tRNAs¹⁰.

All three introns identified so far in eubacteria are in tRNA genes. This may be due to constraints of our detection method (which favours small, abundantly transcribed introns that self-splice efficiently *in vitro*, for example), or introns in tRNA may have been selectively retained during evolution. Although unrelated to group I, introns are frequently present in archaeobacterial and eukaryotic tRNAs, within or next to the anticodon. Deletion of the intron results in failure to modify an anticodon uridine to pseudouridine in eukaryotic tRNA^{Tyr} (refs 18–20). It is possible that introns also have a role in post-transcriptional modification of bacterial tRNAs.

Are group I introns ancient? If introns were present in the common ancestor of all cells, some should have been retained in the eubacterial lineage despite genome 'streamlining'². Closely related introns have now been found in the anticodon loops of tRNA genes from widely divergent bacterial phyla. But as they are in different tRNAs, we cannot determine when these introns were acquired. One intriguing possibility is suggested by the recent demonstration that the aminoacyl tRNA synthetases for Leu, Ile and (unexpectedly) Arg are related²¹, which raises the interesting corollary that their respective tRNAs may also have had a common ancestor. We must therefore consider the possibility that tRNAs were interrupted by introns before completion of the genetic code. Alternatively, the introns may have arrived recently at their present locations, for example by reversal of splicing²² followed by reverse transcription.

Southern hybridization with the *Azoarcus* intron probe (Fig. 4) suggests that related introns are widespread among *Proteobacteria*. As more bacterial introns are sequenced, a comparison of the sequences of introns in homologous genes (for example, for the same tRNA) will provide conclusive evidence concerning the antiquity of group I introns. □

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CORRECTIONS

Evolution of ecological differences in the Old World leaf warblers

Adam D. Richman & Trevor Price

Nature **355**, 817–821 (1992)

IN Fig. 2a of the above letter the second and third rows were inadvertently interchanged. In addition, the sequence at position 15176 (ref. 1) for both *Sylvia* and *P. coronatus* should read 'A' and not 'T'. □

1. Anderson, S. et al. *Nature* **290**, 457–465 (1981).

Structure and expression of a human oxytocin receptor

Tadashi Kimura, Osamu Tanizawa, Kensaku Mori, Michael J. Brownstein & Hiroto Okayama

Nature **356**, 526–529 (1992)

WE have discovered an error in the DNA sequence of our pOTR gene in Fig. 1b. The nucleotides 714–717, CGGC should be GGCGGCG. The amino acids underneath should be Ala Ala Ala instead of Ala Gly. Accordingly, the complementary DNA encodes a 389 amino-acid protein with a relative molecular mass of 42,819. This error does not affect the conclusions of the paper. □

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